INVESTIGATION OF THE MOLECULAR & FUNCTIONAL PATHOPHYSIOLOGY OF POLYCYTHAEMIA VERA

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DECLARATION

I, Tamara Frances Everington, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

This thesis explores the pathophysiological mechanisms underlying the myeloproliferative disease, Polycythaemia Vera (PV). PV is characterised by clonal red cell expansion with a tendency for leukaemic transformation. This study focusses on primary erythroid progenitor cells derived from 27 PV patients and 49 control subjects with non-clonal red cell expansion or normal red cell mass providing insight into survival, signal transduction & the molecular signature of these cells. The erythropoietin independent colonies (EECs) characteristic of PV were detected in 93% PV, the V617F Jak2 mutation was present in 93% PV and marked upregulation of PRV1 mRNA which can be associated with PV was found in 89% PV. None of the control subjects showed these features. PV erythroid progenitors showed increased proliferation with relative resistance to cytokine deprivation when 14 PV samples were compared with 10 controls. Apoptosis was not found to be increased in PV.

In over 40 experiments PV erythroid progenitors showed aberrant signalling with constitutive and stimulated increases in activation of the PI3K, MAPK and Jak/STAT pathways. Activation of each pathway was reduced with specific small molecule inhibitors. Use of PI3K & Jak2 inhibitors caused comparable reduction between PV & control samples in 40 erythroid colony assays and 20 survival experiments using erythroid progenitors. However, there was some evidence to suggest that the Jak2 inhibitors used were less effective in PV with homozygous expression of V617F Jak2. EECs from PV samples were, however, greatly reduced with inhibitors. RNA from 14 erythroid progenitor samples including 6 PV were hybridised onto Affymetrix GeneChip microarrays. There was segregation of signature between subject groups. The array data was validated by real-time quantitative PCR and this technique was further used to show that 2/4 genes identified as upregulated in PV were not Jak2 dependent but that the well recognised Jak/STAT target, Pim-1 was.

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COMMONLY USED ABBREVIATIONS

MPD	Myeloproliferative disease
PV	Polycythaemia Vera
ET	Essential Thrombocythaemia
IM	Idiopathic myelofibrosis
SP	Secondary polycythaemia
IE	Idiopathic erythrocytosis
N	Normal control
Epo	Erythropoietin
EpoR	Erythropoietin receptor
SCF	Stem cell factor
c-KIT	Stem cell factor receptor
IGF1	Insulin growth factor 1
IL3	Interleukin 3
EEC	Erythropoietin independent erythroid colonies
BFUe	Burst-forming unit, erythroid
CFUe	Colony-forming unit, erythroid
JAK	Janus-associated kinase
V617F	Specific point mutation in JAK2 resulting in a valine-phenylalanine substitution at position 617 of the JAK2 protein
WT	wild type form (usually referring to wild type JAK2)
HOM	homozygous (mutant)
Het	heterozygous (mutant)
CD34+	haematopoietic progenitor cell expressing CD34 antigen
PI3K	Phospho-inositide 3 kinase
MAPK	Mitogen-associated protein kinase
STAT	Signal transducer & activator of transcription
PKC	Protein kinase C
PIM1	Pim-1 oncogene (& Pim-1 protein in relation to western blots)
IFITM1	Interferon induced transmembrane protein 1
CBFA2T3	Core binding factor α subunit 2 translocated to 3
LEPR	Leptin receptor

CHAPTER 1 – INTRODUCTION

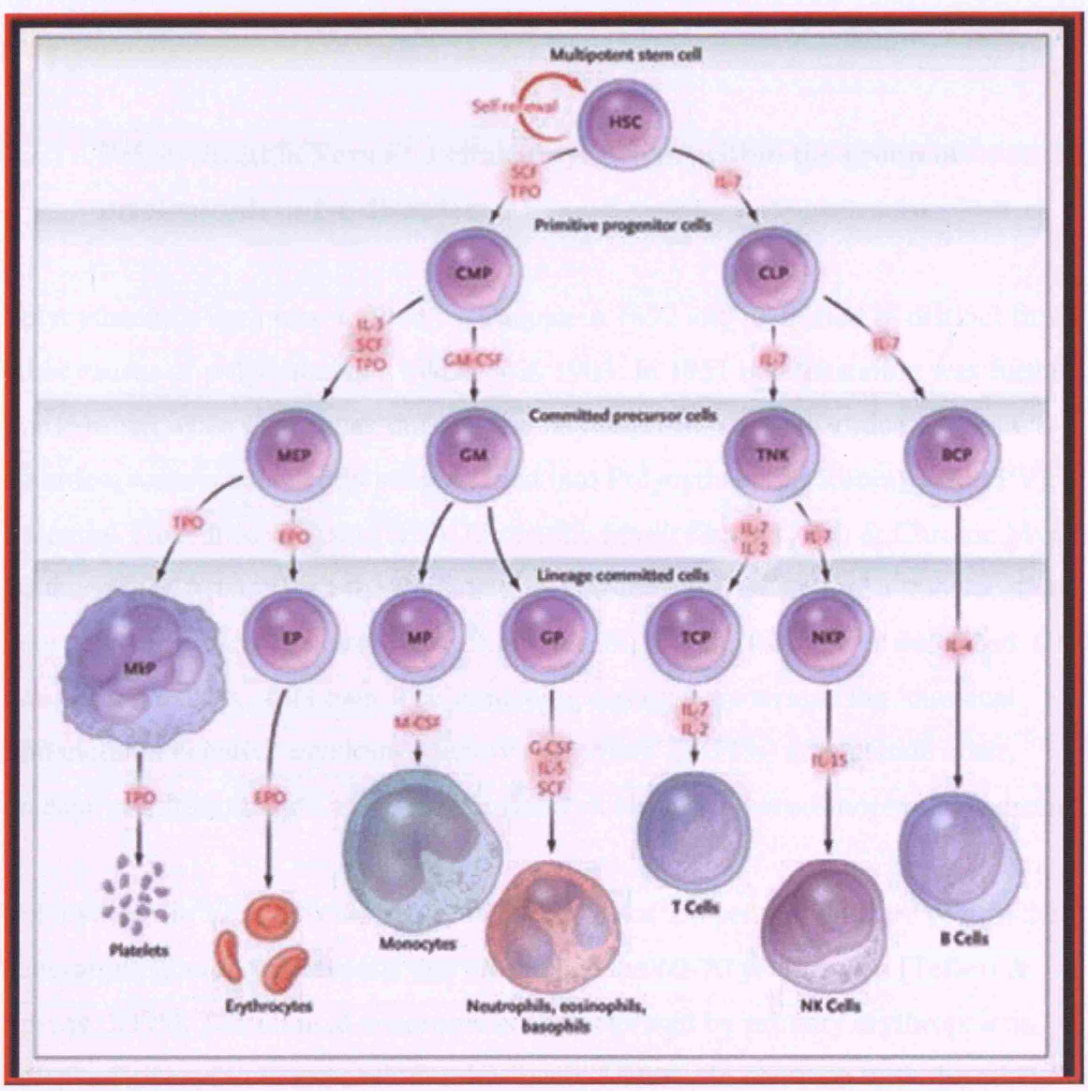
1.1 Normal Haematopoiesis

Haematopoiesis is the process by which multipotent stem cells proliferate and differentiate to produce mature blood cells. It is continually driven by highly co-ordinated patterns of gene expression under the influence of growth factors and hormones. Haematopoietic stem cells are capable of self-renewal and of progression to committed progenitors which can differentiate down the erythroid, granulocytic, monocytic, megakaryocytic and lymphocytic lineages. Haematological diseases such as myeloproliferative disorders or leukaemias are the result of dysregulation at one or more stages of normal haematopoiesis (see Figure 1[1]).

After birth normal haematopoiesis is largely confined to the bone marrow although small numbers of haematopoietic stem cells [HSC] can be found in peripheral blood. The decision as to whether an HSC undergoes self renewal or lineage commitment is governed by the expression of a number of transcription factors and cytokines. Low levels of multiple cytokines maintain basal haematopoiesis with short lived amplifications of specific cytokines boosting production in response to haematopoietic stress. These responses are relevant to haematological disease states and can be exploited to produce committed progenitors in an in vitro setting.

Figure 1[1]: Normal Haematopoiesis

(HSC can self renew and differentiate to common myeloid or lymphoid progenitors [CMP/CLP] under the influence of stem cell factor [SCF], thrombopoietin [TPO] or interleukin-7 [IL-7]. Further differentiation from the CMPs produces myeloid erythroid progenitors [MEP] or granulocytic monocytic progenitors [GM] which further differentiate to progenitors which are megakaryocytic [MkP], erythroid [EP], monocytic [MP] or granulocytic [GP] and go on to produce their mature counterparts. Interleukins-3/5 [Il-3/5], Granulocyte/monocyte colony stimulating factor [GM-CSF, G-CSF or M-CSF] and Erythropoietin [EPO] contribute to these pathways. Further differentiation from the CLPs is through T/Natural killer [TNK] or B cell [BCP] progenitors to mature counterparts supported by interleukins.)



1.2 Background to polycythaemia

As mature red blood cells are the most numerous blood cells in peripheral blood they normally define the relative proportion of blood cells to plasma in terms of volume. This proportion is known as the haematocrit. The haematocrit may be high if the body red cell mass is increased (true polycythaemia) or if the plasma volume is decreased (apparent or spurious polycythaemia). True polycythaemia may occur as a primary abnormality or secondary to haematopoietic stress [SP] such as hypoxia resulting from chronic lung disease. A proportion of patients with true polycythaemia will have primary proliferative polycythaemia (commonly known as Polycythaemia Vera [PV]) due to an an acquired abnormality in haematopoietic stem cells. In many patients with true polycythaemia the cause is not clinically apparent and these patients are labelled as having Idiopathic Erythrocytosis (increased red cell mass of unknown cause, [IE]).

1.2.1 Polycythaemia Vera as a clinical syndrome within the group of myeloproliferative disorders

Polycythaemia Vera was defined by Vaquez in 1892 and identified as distinct from other causes of polycythaemia by Osler in 1903. In 1951 understanding was further transformed when Dameshek defined the 'myeloproliferative disorders'. These disorders were subsequently subclassified into Polycythaemia (Rubra) Vera (PV), Essential Thrombocythaemia (ET), Idiopathic Myelofibrosis (IM) & Chronic Myeloid Leukaemia (CML). When the Philadelphia chromosome created by a translocation between chromosomes 9 and 22 resulting in a BCR-ABL fusion was described, CML moved into a class of its own. The remaining diseases are termed the 'classical Philadelphia negative myeloproliferative disorders' (MPDs) and include other, unusual conditions, such as systemic mastocytosis and hypereosinophilic syndrome.

Polycythaemia Vera develops with an incidence of 2.6 per 100,000 per year in the general population with typical age of onset in the 60-70 years group [Tefferi & Spivak, 2005]. The clinical syndrome is characterized by primary erythropoietin independent erythroid expansion. It has many features in common with the other MPDs including splenomegaly, hyperplasia of the bone marrow, elevation of the

white cell and platelet counts and a predisposition to thrombosis or haemorrhage. PV, in common with ET can transform to IM with at a rate of around 0.3% per annum. Perhaps the most feared complication of all MPDs is the potential for leukaemic transformation. There is a 5 fold increase in the risk of leukaemia development for patients over the age of 70 years with MPDs. This risk is significantly higher for patients who have received alkylating agents or ionising radiation with ^{32}P and much lower for the under 50s. [Marchioli et al, 2005]

Whilst IM can readily be distinguished from PV and IE (idiopathic erythrocytosis) on a bone marrow biopsy, it can be difficult to clinically distinguish ET from PV without performing a red cell mass study. It has recently been recognized that a proportion of patients with ET may represent 'red cell restricted' PV [Campbell et al, 2005] and it now seems likely that PV and ET lie on a diagnostic continuum with co-operating genetic events such as aberrant expression or mutation of the thrombopoietin receptor, c-MPL, contributing to the development of ET [Moliterno et al, 2006; Pardanani et al, 2006].

A major practical issue for haematologists is in differentiating Polycythaemia Vera from the other causes of an elevated haematocrit. In routine clinical practice the most likely cause for an elevated haematocrit is spurious polycythaemia due either to sampling errors or to reduced plasma volume (dehydration). Other patients will have true polycythaemia (but not PV) and are subcategorised into those with Idiopathic erythrocytosis (IE) and those with congenital or acquired secondary polycythaemia (SP). Making the distinction between PV & other conditions is vital because it will alter short, medium & long term clinical management. Key issues here are that the thrombosis incidence of 3.4% is almost certainly higher than that seen in IE or SP, [Pearson et al, 2000] and patients who do not have PV are not at risk of myelofibrotic or leukaemic transformation. From a scientific perspective, accurate diagnosis of PV facilitates understanding of disease pathogenesis, and opens up the potential for development of targetted therapy. As there is no single specific test that defines PV, the Polycythaemia Vera Study Group (PVSG) and World Health Organization (WHO) have defined diagnostic criteria for this disease. These criteria are subtly different and continue to evolve in response to new scientific knowledge. A simplified presentation of these criteria is shown in Figure 2[1].

Figure 2[1]: Polycythaemia Vera Study Group [PVSG] and World Health Organization [WHO] diagnostic criteria for Polycythaemia Vera

PVSG Modified diagnostic criteria for polycythaemia vera [McMullin et al, 2007]

JAK2-positive polycythaemia vera

A1 High haematocrit (>0.52 in men, >0.48 in women) OR raised red cell mass (>25% above predicted)*

A2 Mutation in JAK2

Diagnosis requires both criteria to be present

JAK2-negative polycythaemia vera

A1 Raised red cell mass (>25% above predicted) OR haematocrit >0.60 in men, >0.56 in women

A2 Absence of mutation in JAK2

A3 No cause of secondary erythrocytosis

A4 Palpable splenomegaly

A5 Presence of an acquired genetic abnormality (excluding BCR-ABL) in the haematopoietic cells

B1 Thrombocytosis (platelet count >450 × 109/l)

B2 Neutrophil leucocytosis (neutrophil count > 10 × 109/l in non-smokers, >12.5 × 109/l in smokers)

B3 Radiological evidence of splenomegaly

B4 Endogenous erythroid colonies or low serum erythropoietin

Diagnosis requires A1 + A2 + A3 + either another A or two B criteria

*Dual pathology (co-existent secondary erythrocytosis or relative erythrocytosis) may rarely be present in patients with a JAK2-positive myeloproliferative disorder. In this situation, it would be prudent to Reduce the haematocrit to the same target as for polycythaemia vera

Revised WHO criteria for Polycythaemia Vera [Levine et al., 2007]

Major criteria

Haemoglobin >18.5 g/dl in men, 16.5 g/dl in women, or other evidence of increased red cell volume

Presence of JAK2 V617F or other functionally similar mutation such as the JAK2 exon 12 mutation

Minor criteria

Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation

Serum erythropoietin level below the reference range for normal

Endogenous erythroid colony formation in vitro

Diagnostic

Requires the presence of both major criteria and one minor criterion, or the presence of the first major criterion together with two minor criteria

1.2.2 Polycythaemia Vera as a clonal stem cell disorder

In the 1970s it was recognized that PV results from clonal change in a multipotent haematopoietic progenitor [Adamson et al, 1976]. This clonal change has often been difficult to identify unlike that seen in the related disorder, CML: Standard cytogenetic abnormalities are detected in <20% PV patients and X-linked clonality assays are most informative in younger female patients, who represent a minority of patients with the disease. A major breakthrough in understanding of the clonal nature of the disease came in 2005. Scientific research was published describing a specific G-T point mutation in the pseudo-kinase domain of the Janus-associated kinase 2 (JAK2) gene. This mutation results in a V617F amino acid substitution in JAK2 tyrosine kinase and was found to be highly associated with PV (65-97% cases). The mutation was also found in around half of ET & IM patients [James et al, 2005; Kralovics et al, 2005; Levine et al, 2005; Baxter et al, 2005; Zhao et al, 2005; Jones et al, 2005; Vainchenker et al, 2005].

It is not entirely clear why there is considerable variance (65-97%) in the detection of V617F JAK2 in PV in these studies. Some of the lower levels may be due to 'false negatives' explained by use of different diagnostic criteria for PV and low sensitivity of the mutant screening technique. Some patients with PV may be 'true negatives' with recognized alternative mutations in JAK2 such as those described in exon 12 [Scott et al, 2007]. Others may potentially have mutations in different genes. Equally, there may be false positives within these studies. Whilst early work did not detect the V617F mutation in normal individuals or in patients with IE or SP, this mutation has subsequently been found in healthy individuals when a highly sensitive screening technique has been used: Sidon et al [2006] found the mutation in 5/52 individuals using a technique with sensitivity of 0.01%, Xu et al [2006] found the mutation in 37/3935 normal donors using a technique with a sensitivity of 0.01%. Latterly Passamonti et al [2007] did not find the mutation in 75 outpatients using a technique with 1-2% sensitivity suggesting that this may be an appropriate cut-off level for testing.

V617F JAK2 is uncommon in haematological diseases outwith the MPD category but has been described in Myelodysplastic Syndrome, Leukaemias, Lymphomas &

Myeloma [Steensma et al, 2005; Frohling et al, 2006; Fiorini et al, 2006; Melzner et al, 2006; Lee et al, 2006]. It is now known that the V617F mutation develops in a progenitor cell with lymphoid & myeloid potential but that it is present at low frequency in B & T-lymphocytes and Natural Killer cells [Delhommeau et al, 2007]. This may explain the predominant association of V617F JAK2 with myeloproliferative, rather than lymphoproliferative, disorders. It does not, of course, explain how a single mutation can give rise to 3 distinct conditions; PV, ET & IM, albeit with the highest prevalence in PV.

One explanation for the different MPD phenotypes associated with the V617F JAK2 mutation may be an allele dose effect. Homozygous expression of V617F found in association with the loss of heterozygosity of chromosome 9p is observed in 1/3 patients with PV and is associated with higher haemoglobin [Kralovics et al, 2002]. Homozygosity for V617F JAK2 is, however, unusual in ET [Scott et al, 2006]. Although expression of V617F JAK2 has now been shown to be highly correlated with a diagnosis of PV, it is not yet clear whether or not this mutation may be a primary or secondary event in PV pathogenesis. Some insight into this may be gained by comparing PV patients homozygous & heterozygous for the mutation. Homozygous expression of V617F JAK2 has been shown to be associated with increased duration of disease, [Levine et al, 2005] increased haemoglobin at diagnosis & increased progression to myelofibrosis [Tefferi et al, 2006]. It has not been found to be associated with either the thrombotic or haemorrhagic complications of PV or with the risk of leukaemic transformation [Theocharides et al, 2007]. Indeed there is some evidence to show that V617F JAK2 is not required for leukaemic transformation as wild type JAK2 has been detected in patients who developed acute leukaemia on a background of JAK2 mutant positive PV [Campbell et al, 2006].

If V617F JAK2 was the primary pathogenetic event in acquired PV, erythropoietin independent colonies (EECs) might be expected to be composed solely of JAK2 mutant cells. However, recent data from a group working in the field of PV for many years has identifed wild type JAK2 in PV EECs [Nussenzveig et al, 2007]. If V617F JAK2 was the final pathogenetic event in MPD a clear clonal advantage to the mutant population might be expected but it has been shown in ET that low level clones can be stable at low level over many years [Gale et al, 2007]. It therefore seems likely that

V617F JAK2 is only one of a number of 'hits' required for the full PV phenotype. Studies in familial polycythaemia have detected V617F JAK2 in these patients but found it to be a somatic (acquired) rather than a germline mutation implying that at least one other 'hit' is implicated in this disease also [Rumi et al, 2006].

1.2.3 Polycythaemia Vera and erythropoietin independence

The gold standard test for PV has been the potential for circulating haematopoietic progenitors to develop erythroid colonies in the absence of added erythropoietin (EECs). EECs are not produced from normal individuals, nor from patients with secondary polycythaemia but are found in the majority of patients with PV [Zanjani et al, 1977; Lemoine et al, 1986]. EECs are a highly sensitive diagnostic tool having been detected in patients who had not previously been identified as having PV, but who go on to have an atypical thrombosis which reveals an underlying myeloproliferative disorder [De Stefano et al, 1997; Chait et al, 2005]. EECs were therefore incorporated into both the Polycythaemia Vera Study Group (PVSG) and World Health Organization (WHO) diagnostic criteria for PV (see Figure 1[2]).

The mechanism of EEC formation is not fully understood but it is believed that EECs result from an intrinsic abnormality in progenitor cells as they cannot be abolished by anti-erythropoietin or anti-erythropoietin receptor antibodies [Fisher et al, 1994]. EECs are normally cultured directly from mononuclear cells derived from PV blood or bone marrow but have also been cultured from PV erythroblasts which had previously been differentiated in liquid culture conditions [Silva et al, 1998, Ugo et al, 2004]. Erythropoietin independence is therefore a characteristic feature of PV which is of diagnostic significance and may have functional relevance for maturing erythroid progenitors.

1.3 Signal Transduction in tumour cells & erythroid progenitors

1.3.1 Basic haematopoietic signalling

Intracellular signal transduction is initiated by binding of a ligand to its cognate receptor. This binding triggers recruitment and activation of intracellular molecules which in turn activates downstream targets ultimately resulting in activation of target genes in the cell nucleus. Each signalling pathway can be activated by a spectrum of ligand / receptor associations but this may vary according to the type / dose of ligand. There is overlap and 'crosstalk' between different pathways. Inhibitors may have predominant effects on one pathway but may crossreact with others by either inducing or suppressing activation. The contribution of each pathway to cellular activation varies with cell type. Artificial in vitro systems may not reflect the multiple factors participating in vivo. All of these factors must be taken into consideration when developing new understanding of intracellular signalling mechanisms.

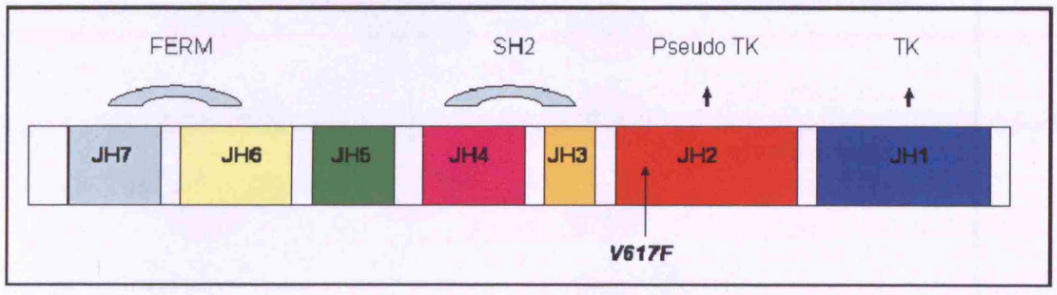
Tyrosine kinase activation is critical to intracellular signalling. Members of the cytokine receptor superfamily such as the Erythropoietin Receptor (EpoR) & the Thrombopoietin Receptor (c-MPL) lack intrinsic tyrosine kinase activity. They activate intracellular signalling by associating with Janus-associated kinases (JAKs) which act as cytosolic tyrosine kinases. Other receptors involved in haematopoiesis which are not part of the cytokine receptor superfamily including the Stem Cell Factor receptor (c-KIT) and the FMS-like Tyrosine Kinase 3 receptor (FLT3) possess a tyrosine kinase domain in their intracellular portion and are therefore theoretically JAK independent.

There are 4 known members of the JAK family, JAK1, JAK2, JAK3 & Tyk2. JAK3 expression is predominantly found in haematological cells but JAKs 1 & 2 and Tyk2 are ubiquitously expressed. JAKs have a unique domain structure amongst tyrosine kinases. There is a C-terminal kinase domain (JAK homology 1 [JH1]) adjacent to a catalytically inactive 'pseudokinase' domain (JH2). The N-terminal region (JH3-4) resembles a Src homology 2 (SH2) domain but its function is not clearly understood & the domain structure is completed by an amino-terminal FERM (band 4.1, ezrin,

radixin, moiesin) domain, JH5-7, which mediates JAK binding to cytokine receptors. [Khwaja A, 2006] JAK2 is known to be critical to erythrogenesis as knockdown of JAK2 resulted in early embryonic lethality in mice due to failed erythropoiesis [O'Shea et al, 2002]. The V617F JAK2 mutation found in PV lies in the JH2 domain within what is believed to be a 'hinge' region of the protein. (see Figure 1[3])

Figure 3[1]: Schematic representation of the JAK2 gene showing the position of the V617F mutation

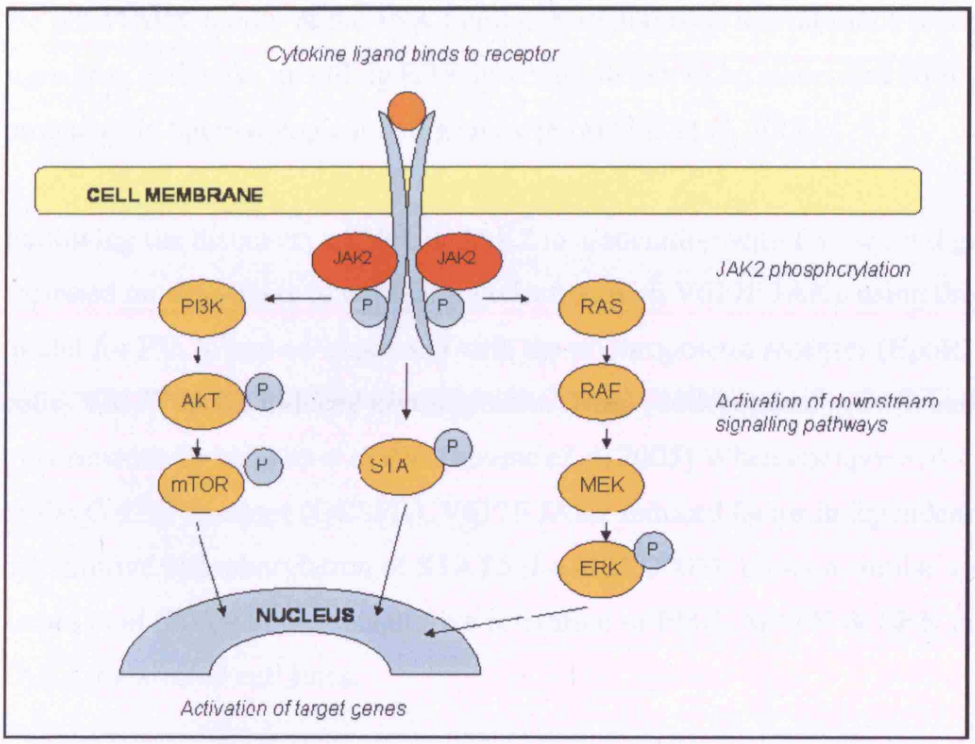
(diagram illustrates the four functional domains of JAK2 including the FERM domain [receptor association], the SH2 domain [phosphotyrosine binding], the JH2 domain [negative regulation of kinase or pseudokinase activity] and the JH1 domain [tyrosine kinase]. The V617F JAK2 mutation lies in the JH2 domain as indicated)



There are several downstream targets of JAKs including the signal transducers and activators of transcription (STATs), the phosphoinositide-3-kinases (PI3K) and the mitogen associated protein kinases (MAPK) [Rane & Reddy, 2002]. This is represented in Figure 1[4]. The JAK / STAT pathway is important for many biological responses including differentiation, proliferation & oncogenesis. STATs are however, not only induced by JAKs, but can also be activated by serine phosphorylation via pathways including MAPK, and by tyrosine phosphorylation independent of JAK [Khwaja, 2006].

Figure 4[1]: Schematic representation of signal transduction via JAK2

(cytokine binds to receptor resulting in association & phosphorylation of JAK2. This then results in recruitment of signal transducer and activator of transcription [STAT] signalling proteins and phosphorylation & activation of downstream signalling pathways including Stat transcription factors, mitogen activated protein kinase [MAPK] signalling proteins and the phospholtidylinositol 3-kinase [PI3K] – AKT pathway)



PI3K are ubiquitously expressed enzymes which are central to cell survival & proliferation and can be divided into 3 classes. Class Ia PI3K consist of a 110 kDa catalytic unit (p110α, p110β or p110δ), and a regulatory 85kDa unit (p85). Class Ib PI3K includes p110γ. These p110 variants are referred to as PI3K isoforms. Expression of PI3K isoforms varies with cell type and there is emerging evidence to suggest that differential inhibition may offer a therapeutic opportunity [Anderson & Jackson, 2004; Billotet et al, 2006]. AKT, a serine / threonine kinase is a key mediator of PI3K signalling and is responsible for phosphorylation of a number of downstream targets including glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR) & p70S6K. There is now overwhelming evidence to show that PI3K, AKT

and downstream targets contribute to the pathophysiology of human tumours [Vivanco & Sawyers, 2002].

MAPK are widely expressed serine / threonine kinases which lie downstream from RAS and have important roles in cell survival & proliferation. RAS mutations resulting in hyperactivation of RAS and its downstream targets have been described in myelodysplastic / myeloproliferative disorders [Side et al, 1998]. MAPK can be divided into 3 different groups; the extracellular signal regulated kinase (ERK) family, the p38MAPK family & the JNK family. Simultaneous activation of several signalling pathways including ERK has been shown to be associated with poor prognosis in haematological malignancy [Kornblau et al, 2006].

Following the discovery of V617F JAK2 in association with PV, several groups have focussed on the effects of cellular transfection with V617F JAK2 using this as a model for PV. When co-expressed with the erythropoietin receptor (EpoR) in BAF3 cells, V617F JAK2 induced erythropoietin (Epo) - independent growth and Epo hypersensitivity as is seen in PV. [Levine et al, 2005] When coexpressed with c-MPL or the G-CSF receptor (GCSFR), V617F JAK2 induced factor independence and constitutive phosphorylation of STAT5 [Lu et al, 2005]. Using a similar approach James et al [2005] noted constitutive activation of PI3K, MAPK & ERK in V617F JAK2 transfected cell lines.

1.3.2 Erythropoietin, stem cell factor & erythroid signalling pathways

Erythropoiesis is regulated by a number of growth factors with Erythropoietin (Epo) and Stem cell factor (SCF) playing central roles. Committed erythroid progenitors with Burst Forming Units – Erythroid (BFUe) & Colony Forming Units – Erythroid (CFUe) potential are initially produced without Epo but definitive erythropoeisis (proliferation, survival & differentiation) cannot normally take place in the absence of Epo or EpoR [Wu et al, 1995; Lin et al, 1996]. SCF has also been shown to be critical to erythropoiesis as deletion of c-KIT leads to anaemia in mice resulting in embryonic lethality [Munugalavadla V & Kapur R, 2005].

When SCF binds to c-KIT the receptor dimerises & autophosphorylates. This creates binding sites for a variety of SH2 domain-containing enzymes and adaptor proteins such as the p85 component of PI3K, Ras GTPase activating protein & Src kinases. When Epo binds to EpoR, the associated JAK2 tyrosine phosphorylates the receptor [Munugalavadla V & Kapur R, 2005]. Receptor activation creates binding sites for SH2 domain-containing proteins including STAT5 and the p85 component of PI3K [Munugalavadla V & Kapur R, 2005]. It can therefore be seen that SCF & Epo signal via different mechanisms but show overlapping downstream effects.

The precise physiological role of each signalling pathway in erythropoeisis is not fully elucidated. The PI3K pathway via phosphorylation of AKT and its downstream target GSK3 has been shown to be critical to erythroid proliferation, differentiation & survival. [Haseyama et al, 1999; Somervaille et al, 2001] The MAPK & STAT pathways have individually and collaboratively been shown to support erythropoiesis & erythroid expansion [Sui et al, 1998; Sasaki et al, 2000].

It is difficult to separate out the contributions of Epo & SCF to individual signal pathway component in erythroid cells. Taken simplistically, Epo (but not SCF) directly induces phosphorylation of STAT5 via JAK2 in erythroid progenitors. Epo also transiently induces phosphorylation of ERK in normal erythroid cells. SCF acts as the major contributor to phosphorylation of AKT in early erythroid progenitors (although Epo has some independent effect), but also sustains phosphorylation of ERK in these cells [Arcasoy & Jiang, 2005].

1.3.3 Effect of inhibition of signalling pathway components on erythropoiesis

Blockade of each of the PI3K, MAPK & STAT5 pathways has been shown to affect erythroid signalling. LY294002 is a small molecule PI3K inhibitor which has been shown to reduce phosphorylation of AKT and to reduce the Epo & SCF supported proliferation and maturation of erythroblasts [Myklebust et al, 2002].U0126 is a small molecule MAPKK inhibitor which has been shown by some [Mori et al, 2003] though not by others [Somervaille et al, 2001] to affect erythroid survival. Absence of STAT5 in mice has been shown to reduce erythroblast survival & differentiation. [Sokolovsky et al, 2001].

1.4 Erythroblast proliferation & survival

1.4.1 Aberrant proliferation in Polycythaemia Vera

Erythropoietin independent colonies are highly associated with a diagnosis of PV but Epo-independence is not due to mutations in either Epo or EpoR. [Hess et al, 1994] Epo levels are lower in untreated PV than in control subjects but there is evidence to suggest that treatment of PV with phlebotomy or hydroxyurea is associated with relatively higher levels of Epo, with hydroxyurea having the greater effect [Shih et al, 1998; Andreasson et al, 2000; Johansson et al, 2006]. This suggests that the low levels of Epo observed in PV occur as a result of physiological feedback.

The haematopoietic expansion observed in PV in vivo occurs despite reduced levels of Epo. One explanation for excessive proliferation in PV could be hypersensitivity to Epo and indeed this has been described [Montagna et al, 1994]. However, hypersensitivity to Interleukin-3 [Dai et al, 1991], Granulocyte/Monocyte-Colony Stimulating Factor [Dai et al, 1992], Stem Cell Factor [Dai et al, 1994] & Insulin Growth Factor-1 [Correa et al, 1994] has also been reported. Therefore increased proliferation in PV is neither cytokine specific nor restricted to those cytokines known to activate signal transduction predominantly via JAK2.

1.4.2 The effects of JAK2 inhibition on erythropoiesis

Small molecule inhibitors of JAK2 include the tyrphostins (AG490), the indolocarbazoles (Staurosporine, Go6976) and a pyridine containing tetracycle called Compound 6 (also known as Jak Inhibitor 1). None of these groups of inhibitors are absolutely specific for JAK2. Tyrphostins are protein tyrosine kinase inhibitors that were initially explored as inhibitors of epidermal growth factor expression. [Margolis et al, 1989] They were subsequently found to also inhibit cyclin dependent kinases [Kleinberger-Doron et al, 1998] Indolocarbazoles were described as inducers of DNA topoisomerase I [Yamashita et al, 1992] before their inhibitory effects on Protein Kinase C (PKC) [Martiny-Baron, 1993] and later, JAK2 & FLT3, [Grandage et al, 2006] became apparent. Jak Inhibitor 1 was more recently described as a Jak inhibitor with predominant effects on JAK2 & Tyk2 but some effect on JAK1 & JAK3. [Thompson et al, 2002]

Inhibitors of JAK2 have been tested in tumour cell lines and have generally been shown to reduce viable cell number and cell growth. Thompson et al [2002] found that Jak Inhibitor 1 reduced proliferation in a murine T-cell lymphoma cell line, Spiekermann et al [2001] detected growth arrest with AG490 in AML cell lines and Iankov et al [2002] found that Go6976 blocked proliferation in a plasmacytoma cell line. Work in primary cells has also consistently shown that JAK2 inhibition reduces cell growth. Meydan et al [1996] found that AG490 reduced the proliferation of primary acute lymphoblastic leukaemia cells in vivo and in vitro. Grandage et al [2006] described reduced proliferation of primary acute myeloid leukaemia cells by Go6976 in a PKC independent fashion. Ugo et al [2004] found that AG490 reduced erythroblast survival and differentiation.

Transplantation studies in murine models have shown that bone marrow progenitors transfected with V617F JAK2 induce erythroid expansion [James et al, 2005] and that this expansion can be reduced by inhibitors of JAK2 [Zaleskas et al, 2006]. Some groups have looked at whether JAK2 inhibitors might have differential effects on V617F JAK2 mutant compared to wild type JAK2 expressing tumour cells. Walz et al [2006] compared effects of Jak Inhibitor 1 on HEL cells (V617F JAK2) and K562 cells expressing transformed BCR-ABL (but wild type JAK2). They found reduced

cell growth in the HEL cells but not in the K562 cells. Interestingly apoptosis as assessed by Annexin V staining was only minimally increased in the Jak Inhibitor 1 exposed HEL cells with no effect seen on the control K562 cells. Walters et al [2006] similarly found a greater reduction of cell growth in V617F JAK2 expressing cell lines (HEL, SET-2, UKE-1) than in wild type JAK2 controls (K562, SKM). These findings suggested that mutant JAK2 may be more sensitive to inhibition than wild type JAK2.

Kawada et al [1997] recognized that Staurosporine could reduce erythroid colony formation from peripheral blood and bone marrow. These authors found that the 50% inhibitory concentrations (IC50) for Staurosporine were higher in PV than in normal controls. If we assume that the PV cells used in these experiments were V617F JAK2 mutant cells, this would imply reduced sensitivity of primary mutant cells to inhibition. Jamieson et al [2006] investigated the effects of AG490 on erythroid colony formation in PV & normal controls and found that erythroid colonies were reduced by JAK2 inhibition. However, this inhibition preferentially affected normal colonies as V617F mutant colonies were not reduced in 3/4 PV patients. This evidence in primary cells suggests that the V617F JAK2 mutation may confer relative resistance to JAK2 inhibition.

1.4.3 The effects of PI3K inhibition on erythropoiesis

The PI3K system is highly complex. LY294002 is a potent small molecule pan-PI3K inhibitor with an IC50 of 1.4uM and its effects have been widely studied [Vlahos et al, 1994]. LY294002 has been shown to reduce the Epo & SCF supported survival of normal erythroblasts [Somervaille et al, 2001; Myklebust et al, 2002]. Latterly it has become clear that LY294002 also inhibits Pim-1 (a downstream target of JAK/STAT) and Casein Kinase 2 (CK2) [Jacobs et al, 2005] and so alternative and more specific inhibitors of PI3K and downstream targets are being developed.

As yet there is little published data on the effects of PI3K inhibitors on survival in PV erythroid progenitors: Ugo et al [2004] observed similar effects of PI3K inhibition on erythropoietin dependent and independent erythroblast differentiation in PV but found little impact on erythroid progenitor survival.

1.5 Gene expression in erythroid progenitors

1.5.1 Gene expression studies in Polycythaemia Vera

Gene expression profiling could theoretically contribute to the understanding of disease by offering a diagnostic tool, in the sub-classification of disease entities with potential for prognostic impact and in identification of genes which may be involved in the molecular pathophysiology of disease. Gene profiling of progenitor cells has been extensively explored in haematological malignancy and is well described as a classification tool in Idiopathic Myelofibrosis [Jones et al, 2005; Guglielmelli et al 2007]. To date there is little published data on expression profiles in Polycythaemia Vera progenitors.

Three separate groups have published data on the gene expression profile of neutrophils in PV [Pellagatti et al, 2003; Goerttler et al, 2005; Kralovics et al, 2005]. Pellagatti et al compared profiles between 11 PV & normal controls and found 147 genes with >2.5x upregulation in PV of which 11 were upregulated in all 11 PV patients representing a possible gene signature. These upregulated genes included protease inhibitors known to inhibit neutrophil apoptosis, and other antiapoptotic and survival factors. Goerttler et al compared profiles between 40 PV and 12 SP and found that 64 genes effectively differentiated the signatures. They noted that 253 genes were >1.5x upregulated & 391 downregulated in PV and went on to focus on the upregulation of transcription factors including NF-E2. Kralovics et al examined profiles in 98 patients with PV (54 PV, 33 ET, 11 MF), 4 with SP, 4 with reactive neutrophilia, 3 G-CSF mobilised normal subjects and 28 normal controls. They found 280 genes to be significantly dysregulated in MPD; however the 13 most predictive genes and 3 others shown to be affected were also G-CSF inducible in subjects with wild type JAK2 limiting the pathophysiological interpretation of this data. Significantly, looking at the data presented, there was no commonality in altered gene expression profiles between the studies.

One group has compared the expression profiles of PV CD34+ progenitor cells derived from bone marrow with CD34+ bone marrow cells from normal volunteers

[Steidl et al, 2005]. They found differential expression of 107 genes describing downregulation of pro-apoptotic genes and upregulation of fibrosis stimulating growth factors in PV. They also identified upregulation of a number of receptors, protein kinases & proteasome components which, they observed, might represent therapeutic targets in PV and went on to focus on the retinoic acid receptor components and the potential treatment role of all-trans retinoic acid (ATRA).

Polycythaemia Vera is a clonal myeloid disorder and, in the case of the V617F JAK2, it has been shown that this mutation can be detected from lymphomyeloid progenitors to each of their mature progeny. [Delhommeau et al, 2007] This finding supports the diagnostic relevance in PV of screening gene expression in myeloid cells, whether these are neutrophils or progenitor cells. Polycythaemia Vera is however, by definition, a disorder with predominant effects on the erythroid lineage and it is this feature which discriminates PV from other myeloproliferative disorders. Committed erythroid progenitors have distinct expression profiles and, as might be predicted, the gene expression of committed erythroid progenitors differs from that of other lineage-specific progeny from common CD34+ stem cells [Gubin et al, 1999; Komor et al, 2005]. It has been suggested that the erythroid expansion which predominates in PV might be due to co-operating genetic events not related to the V617F kinase activity [Vainchenker et al, 2005]. As yet there is no published information on the gene expression profiles of committed erythroid progenitors in PV which might indicate such co-operating genetic events in these cells.

The aim of this thesis was to explore the functional and molecular changes apparent in erythroid progenitors from a well characterised cohort of patients with Polycythaemia Vera. Comparison was to be made with erythroid progenitors derived from patients with Idiopathic Erythrocytosis, Secondary Polycythaemia and normal controls. Functional assessments were to include analysis of growth and survival, exploring the signalling platforms underlying these processes. Molecular assessments were to establish a 'snapshot' of erythroid progenitor gene expression using a broad expression profiling tool and selected single gene analysis. The therapeutic potential of small molecule inhibitors was to be tested by introduction into each of these systems.

CHAPTER 2 – METHODS

2.1 The Polycythaemia Clinic

The Polycythaemia Clinic at University College Hospital, London accepts referrals of patients with elevated haematocrit from general practitioners and other hospitals in the North Thames region. All patients were clinically assessed, investigated, diagnosed and managed according to standard protocols by myself [McMullin et al, 2005]. Informed consent was obtained for processing and storage of patient samples. Research results were discussed with patients.

2.1.1 Clinical profiling

A standard clinical history and examination was performed on all patients which included specific questions about symptoms or signs which might be associated with PV and those which might suggest an alternative cause for an elevated haematocrit.

2.1.2 Routine laboratory profiling

Full blood count with differential was performed on at least 3 occasions and a blood film reviewed. Routine biochemistry was performed to check renal and liver function searching for secondary causes of an elevated haematocrit and as a clinical baseline against further therapy. ESR, urate and serum vitamin B12 were checked as low ESR, high urate and high vitamin B12 are often observed in PV. Serum erythropoietin was measured by ELISA as low levels are highly associated with a diagnosis of PV and high levels are suggestive of a secondary cause for polycythaemia.

Patients with a sustained increase in haematocrit (but <0.58 for females and <0.6 for males) underwent radio-isotopic investigation for assessment of red cell mass and plasma volume if there was no clinical imperative for early treatment. True polycythaemia was defined where red cell mass was >25% above the mean of the normal predicted value for weight. Borderline cases were noted and the diagnosis kept under regular review. One borderline patient went on to develop PV.

Pseudopolycythaemia was diagnosed where plasma volume was low or normal and red cell mass normal and these patients were not investigated further: consideration was given to the body mass index in individual patients as obesity can give spuriously low prediction of red cell mass as assessed by weight in these circumstances.

Capillary oxygen saturation at rest and on exercise was performed to assess respiratory function. Younger patients or patients in whom the clinical diagnosis was unclear were encouraged to have bone marrow assessment by aspirate with cytogenetic analysis and trephine with histopathological review. Chest X-ray and abdominal ultrasound scanning was performed as a search for a secondary cause for polycythaemia and to look for the presence of splenomegaly. Further investigations were performed as necessary in individual patients to explore causes for secondary or constitutive polycythaemia.

A diagnosis of Polycythaemia Vera was made at the time of this study according to the Polycythaemia Vera Study Group (PVSG) criteria [Pearson & Messinezy, 1996]. These criteria have subsequently been updated and are shown in Figure 1[2]. The change in criteria did not alter the diagnosis of any individual assessed in this study. The remaining patients with true polycythaemia were classified as having secondary polycythaemia or idiopathic erythrocytosis according to individual clinical and laboratory parameters.

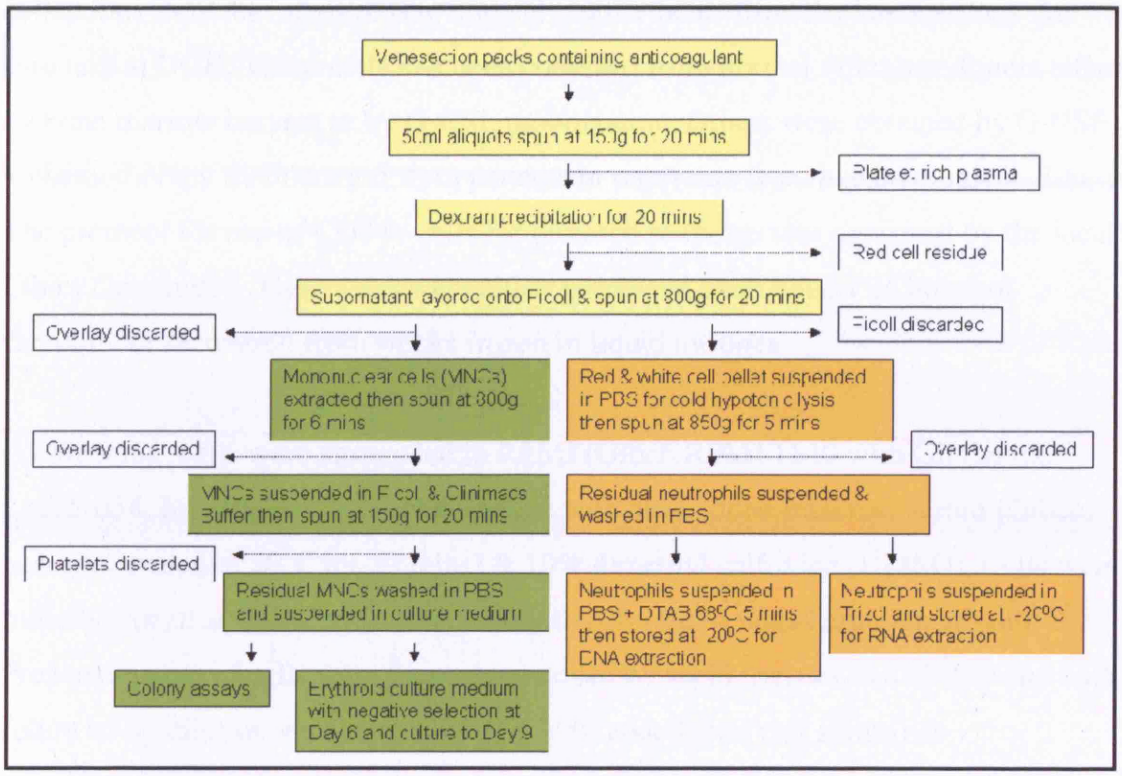
2.1.3 Venesection procedure

Therapeutic venesection was performed with a clean technique by specialist haematology nurses on the haematology day care unit. As per guidelines patients were venesected if their haematocrit exceeded threshold (0.45 for PV, 0.5 for IE/SP & Pseudopolycythaemia). Approximately 450ml venous peripheral blood was taken into "wet packs" (CPDA-1 single blood collection system, R8104 containing 63ml CPDA-1 anticoagulant solution, Baxter).

2.2 Processing and liquid culture of primary haematopoietic cells

Figure 1[2]: Flow chart for initial processing of primary cells

(this chart outlines the first steps of cell separation from the day of sampling with the methodological detail defined in the subsequent text)



2.2.1 Sources and initial storage of primary haematopoietic cells

Venesection packs from patients attending the Polycythaemia Clinic were the principal source of primary cells. Packs were taken from patients with PV, IE and Pseudopolycythaemia. The majority of venesection samples were held at room temperature before processing the same day. A few samples received late in the day were stored at 4^0C overnight and allowed to warm to room temperature prior to processing the next day.

Normal peripheral blood was donated by volunteers within the Department of Haematology at UCL. Some normal cells were obtained as 'Buffy Coat Residues' supplied by the National Blood Service (In order to produce platelets the NBS pools 4

normal donor buffy coats. Platelets were removed from these buffy coats by slow centrifugation leaving a highly cellular buffy coat residue rich in white cells.) These Buffy Coat Residues were delivered on the same day as sampling. Normal cells were stored and processed in the same way as patient samples.

A third source of 'normal' control cells for experiments was CD34+ rich cell collections available as surplus to clinical requirements from the haematology day care unit at UCH. These cells had been collected from normal volunteer donors either by bone marrow harvest or by G-CSF mobilization. Others were obtained by G-CSF + chemotherapy mobilization from patients in remission from haematological disease. The protocol for use of CD34+ cells for research purposes was approved by the local Ethics Committee. These cells were either processed fresh within 24 hours of sampling or recovered from stocks frozen in liquid nitrogen.

For freezing, cells were suspended in RPMI (Gibco RPMI 1640 with Glutamine, 21875-034, Invitrogen Life Technologies) with 50% added fetal calf serum (Gibco, heat inactivated at 56^0C for 30 mins) & 10% dimethyl sulfoxide (DMSO). Cells were initially frozen at -80^0C and subsequently transferred to liquid nitrogen at -180^0C. Frozen aliquots of cells were thawed as needed by rapid transfer to a 37^0C water bath followed by dilution with R10, (RPMI + 10% added fetal calf serum) & centrifugation at 350g. A single wash in phosphate buffered saline (Gibco D-PBS – $CaCl_2$ –$MgCl_2$, 14190-094, Invitrogen Life Technologies) was performed prior to resuspension in the appropriate culture medium.

2.2.2 Manual separation of cells from peripheral blood

All processing was performed in a laminar air flow cabinet with sterile instruments and reagents. Anticoagulated blood was decanted into 50ml containers using sterile transfer units (Plasma transfer set with coupler and Luer adaptor, VMC2240, Baxter). Samples from patients with markedly elevated haematocrit were diluted 1:1 with sterile PBS. Buffy coat residues were diluted 1:5 as the haematocrit of these packs was very high. Samples were centrifuged at 800 rpm/150g for 20 minutes following which the overlying platelet rich plasma was aspirated and discarded.

Red cell depletion was then performed by dextran precipitation. Five millilitres of an isotonic 10% dextran solution (25g Dextran, 17-0320-02, Amersham Biosciences with 2.25g NaCl in 250ml deionised distilled water [DDW], autoclaved) was added to 45ml of cells. Mixing was performed by gentle inversion and uncapped samples were left to stand for 20 minutes following which the red cell depleted supernatant was harvested. This supernatant was then carefully layered in 25ml aliquots onto 20ml Ficoll-Paque TM Plus (17-1440-03, Amersham Biosciences) in 50ml containers. These samples were then centrifuged at 1800 rpm/800g for 20 minutes.

Following centrifugation approximately 20ml supernatant was aspirated and discarded. The mononuclear cell layer was carefully aspirated into a fresh container. The remaining Ficoll was aspirated and discarded and the red cell + granulocyte pellet was suspended in 2.5ml PBS before further processing.

The mononuclear cell layer was centrifuged at 1800rpm/800g for 6 minutes to remove residual Ficoll. The residual pellet was then resuspended in 5ml Ficoll and made up to 50ml with CliniMACS Buffer (700-25, Miltenyi Biotec). Samples were centrifuged at 800rpm/150g for 20 minutes and the supernatant containing residual platelets was aspirated and discarded. Mononuclear cells were suspended in 50ml PBS and centrifuged at 1300rpm/350g for 5 minutes to remove residual Ficoll then resuspended in R10. The number of residual viable cells was counted by addition of 0.4% Trypan blue solution (T8154, Sigma) followed by enumeration with the aid of a haemocytometer.

2.2.3 Purification & storage of neutrophils from peripheral blood

The red cell + granulocyte suspension derived in 2.2.2 was subject to controlled cold hypotonic lysis to destroy residual red cells: 8ml of deionised distilled water (DDW) at 4^0C was added to the suspension, inverted to mix and allowed to stand for 1 minute. Residual cells were rescued by addition of 4ml of 3.5% NaCl chilled to 4^0C. The sample was centrifuged at 2000rpm/850g for 5 minutes. Lysed red cells were rinsed off with PBS and the residual pellet was suspended in 10ml PBS. A cell differential was performed with an automated cell counting system (Sysmex) to document that the cellular population was >95% neutrophils.

Neutrophils for future RNA extraction were suspended at a concentration of 10 million / ml in Trizol Reagent (15596-026, Invitrogen) then stored at -20^{0}C. Neutrophils for subsequent DNA extraction were suspended in 2.5ml PBS, made up to 7ml with 7.5M DTAB solution (DTAB, D-8638, Sigma 20g + NaCl 22g + 1M Tris solution, pH 8.6, 25ml + 0.5M EDTA solution 25ml + up to 250ml with DDW) , mixed, incubated at 68^{0}C for 5 minutes in a waterbath and then stored at -20^{0}C.

2.2.4 Liquid culture of mononuclear cells promoting erythroid expansion & differentiation

Mononuclear cells derived in 2.2.2 were suspended at a density of 2.5-5 x10^6/ml in a medium which differentially promoted erythroid differentiation and amplification over other myeloid lineages. (Gibco IMDM, 21980-032, Invitrogen Life Technologies 40ml + fetal calf serum 10ml + human SCF, 300-07, Peprotech Inc at 10ug/ml + human IGF-1, 100-11, Peprotech Inc at 40ng/ml + recombinant human erythropoietin, Eprex, Janssen-Cilag Inc at 1iu/ml + recombinant human Interleukin 3, I 4144, Sigma at 0.5ng/ml + Beta Estradiol E2257, Sigma at 0.15uM + Dexamethasone at 1uM). Samples were incubated in tissue culture flasks at 37^{0}C 5% CO_2 for 6 days.

2.2.5 Negative selection of mononuclear cells & subsequent culture

Day 6 cells (2.2.4) were carefully aspirated to avoid dislodgement of adherent macrophages. Aspirated cells were counted and centrifuged at 1300rpm for 5 minutes. Cells were suspended in CliniMACS buffer (see 2.2.2) which had been stored at 4^0C and then incubated for 15 minutes at room temperature with microbeads conjugated to antibodies to CD2, CD14 & CD19 at 20ul per 10^7 cells (human CD2 MicroBeads 130-091-114, human CD14 MicroBeads 130-050-201, human CD19 MicroBeads 130-050-301, Miltenyi Biotec).

Following incubation, cells were washed to remove unbound antibody and resuspended in fresh CliniMACS buffer. Samples with cell number $>5x10^7$ then underwent selection with the VarioMACS system (LS Separation Column, 130-042-401 + Pre-separation filter, 130-041-407, Miltenyi Biotec). Samples with cell number $<5x10^7$ then underwent selection with the MiniMACS system (MS Separation Column, 130-042-201 & Filter, Miltenyi Biotec). The unbound fraction was collected and passed down a second column. The final unbound fraction was collected, centrifuged at 1300rpm/350g for 5 minutes and suspended at a density of 0.2-$1x10^6$/ml in the erythroid differentiating medium described in 2.2.4 but without added Estradiol or Dexamethasone.

The negatively selected population was then cultured at 37^0C 5% CO_2 for 3 days in tissue culture flasks. If the cells contained residual non-erythroid precursor cells by visual inspection, they were reselected or adhesion-depleted in the case of macrophage contamination. If there was excessive debris or red cell contamination the samples were ficolled to remove these unwanted components. Unless specifically stated otherwise cells were used for experiments on Day 9 of culture as this was found to be optimal in terms of cell number and homogeneity of the cellular population.

2.2.6 Erythroid differentiation of CD34+ rich samples

Progenitor rich samples derived as per 2.2.1 were positively selected up front to obtain a pure population of CD34+ cells. Cells were suspended in CliniMacs Buffer and incubated for 15 minutes with human CD34+ microbeads (130-046-702, Miltenyi-Biotec) according to the manufacturer's instructions. A washing step was performed and cells were passaged down a magnetic column with filter using the VarioMacs sytem. The CD34+ fraction was then collected according to manufacturer's instructions and cells were incubated for 6-11 days in erythroid medium as per 2.2.4. Derived cells were 80-90% erythroblasts at this stage with 10-20% non-erythroid myelomonocytic precursors as assessed by May-Grunwald-Giemsa staining of cytospins.

2.2.7 Evaluation of samples by morphological analysis

A standard cytospin using $5x10^4$ cells in 75ul medium was performed. Dried samples were fixed, stained & counterstained using methanol and the May-Grumwald-Giemsa system and then microscopically evaluated. Day 9 cells were deemed suitable for microarray analysis if they were >95% pure erythroblasts or suitable for other work if they were >90% pure erythroblasts. CD34+ samples were used if the population was >80% erythroblasts.

2.2.8 Evaluation of samples by immunophenotyping

Immunophenotyping was performed on the derived cells by flow cytometric counting (Epics Elite, Beckman Coulter, High Wycombe, UK) following labelling with Phycoerythrin or FITC-conjugated antibodies to Glycophorin A, CD36, CD117, CD71 and IgG (PE anti-human Glycophorin A, 555570, PharMingen & FITC anti-human CD36, 555454, PharMingen & anti-human CD71/FITC, F0829, DakoCytomation & Human CD117, CD11704-4, Caltag Laboratories & mouse anti IgG-FITC 345815, Becton Dickinson & normal mouse IgG1 PE, sc-2866, Santa Cruz Biotechnology). Samples were deemed suitable for further experiments if (in addition to morphological analysis) they showed intermediate positivity for Glycophorin A, strong positivity for CD36 & CD71 and weak expression of CD117.

2.3 Colony assays

Colony assays were performed in triplicate with $1x10^5$/ml mononuclear cells in semi-solid culture medium aliquoted into 24-well tissue culture plates. Erythroid (both BFUe and CFUe) and granulocyte-macrophage colonies were enumerated microscopically after 13-14 days of culture in a humidified atmosphere at 37^0C, 5% CO_2. Equivocal samples were reviewed by an independent assessor.

2.3.1 Standard colony system

Standard colony assays were performed as above using aliquots from a 100ml stock made up of 80ml Methocult (H4230, Stem Cell Technologies) + 20ml IMDM +30ng/ml human IL3 + 1iu/ml Erythropoietin + 10ng/ml SCF (see 2.2.4) + 25ng/ml G-CSF (Filgrastim-Neupogen, Amgen) and 25ng/ml GM-CSF (gift from Hoechst, UK).

2.3.2 Erythropoietin independent colonies (EECs)

Erythropoietin independent colony assays were performed as above using Methocult without added erythropoietin (H4531, Stem Cell Technologies). Erythropoietin was added to this medium at concentrations of 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03 and 0 iu/ml.

2.3.3 Colonies with added inhibitors & cytokines

The effect of small molecule inhibitors on colony assays was assessed by addition of specified concentrations of inhibitor or cytokine to colony assay media (either standard or EECs) prior to plating.

The following cytokines were used:

Interferon alpha (Roferon, Roche Pharmaceuticals)
Interferon gamma (I3265, Sigma)
Recombinant Human Leptin (300-27, PeproTech EC)

The following inhibitors were used:

PI3 kinase inhibitor: PI103 (528100, Calbiochem)

Jak 2 inhibitors: Go6976 (365250, Calbiochem), Jak Inhibitor 1 (420097, Calbiochem)

PKC inhibitor: Go6983 (365251, Calbiochem)

mTOR inhibitor: Rapamycin (R5000-5M, LC Laboratories)

2.4 DNA & RNA extraction & processing

Samples suitable for DNA or RNA extraction were stored at -20^{0}C prior to processing.

2.4.1 DNA extraction chloroform technique

Samples from 2.2.3 were thawed at room temperature prior to addition of an equal volume of Chloroform (100776B, AnalaR). Vigorous mixing was performed prior to centrifugation at 3000rpm for 20 minutes at 4^{0}C. The upper layer was then carefully removed to a clean tube, an equal volume of 100% Ethanol (10107, AnalaR) was added and the sample was gently mixed. Precipitated DNA was transferred to a 1.5ml centrifugation tube and washed with 0.5ml 70% Ethanol, then centrifuged at 3000rpm for 5 minutes. Residual ethanol was removed and the DNA pellet was suspended in DDW. To ensure solubilization, samples were left on a rotating shaker at 4^{0}C overnight prior to storage at 4^{0}C.

2.4.2 RNA extraction chloroform technique

Trizol samples (0.5ml) from 2.2.3 were thawed at room temperature prior to addition of 200ul Chloroform. Vigorous mixing was performed prior to centrifugation at 13,000rpm for 15 minutes at 4^{0}C. The upper layer from this spin was then carefully aspirated and transferred to a fresh container. Five hundred microlitres of Propan-2-ol (102246L, AnalaR) was added and the samples incubated for 10 minutes at room temperature prior to centrifugation at 13,000rpm for 10 minutes at 4^{0}C. The supernatant was aspirated and discarded and the RNA pellet washed with 1ml 70% Ethanol prior to centrifugation for 5 minutes at 4^{0}C. Residual ethanol was removed and the RNA pellet was suspended in DDW. To ensure solubilization, samples were left on a rotating shaker at 4^{0}C overnight prior to storage at -20^{0}C.

2.4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

Oligo dTs were diluted in sterile water to a final volume of 8.8ul and 2ul of the RNA extracted as per 2.4.2 was added to this. Denaturation was performed at 65°C for 5 mins followed by cooling to room temperature. Reverse transcription was then performed by addition of 9.2ul of a mixture consisting of 2ul 10 x NH_4 Reaction Buffer (Bioline) + 4.2ul 25mM $MgCl_2$ (Bioline) + 2ul 10mM dNTPs (Bioline) + 0.5ul RNAse Inhibitor (N2611, Promega) + 0.5ul AMV Reverse Transcriptase (M5108, Promega). Mixed samples were incubated at 42^0C for 1 hour for reverse transcription to occur followed by incubation at 95^0C for 5 minutes to terminate the reaction. cDNA was stored at 4^0C pending subsequent use or used immediately for PCR reactions.

2.4.4 Semi-quantitative analysis of transcripts of PRV1 and other genes

A multiplex PCR reaction for PRV1 with GAPDH as a housekeeping gene was performed within the same tube to allow semi-quantitative analysis of products. An 18ul mix consisting of 1.8ul 10 x NH_4 Reaction Buffer (see 2.4.3) + 0.2ul 10mM dNTPs + 0.4ul 25mM $MgCl_2$ (see previous) + 12.6ul sterile water + 1ul Biotaq DNA Polymerase (BIO-21040, Bioline) + 0.5ul each of forward and reverse primers for PRV1 (forward 5'-ATTGAGAGCGGACCCCAAGTGA-3', reverse 5'-CTTGGTATTCGATGTGGTCCA-3') & GAPDH (forward 5'-GCCGAGCCACATCGCTCAGA-3', reverse 5'-GAGGCATTGCTGATGATCTTG-3') was added to 2ul sample cDNA (from 2.4.3). PCR was performed as follows; 95°C for 5 minutes, then 30 cycles of 95°C for 30 seconds + 64°C for 30 seconds + 72°C for 30 seconds followed by completion with 1 cycle of 72°C for 10 minutes. PCR products were mixed with 3ul 6x Loading Dye (R0611, Fermentas) and run on a 1.5% agarose gel with added Ethidium Bromide to allow separation and visualisation of products. Bands were referenced to those for 2 control samples in which PRV1 expression had previously been ascertained; one with overexpression and one with low expression of PRV1. In comparison with an adequate GAPDH band, PRV1 expression was said to be absent if no band of the correct size was seen, low level if a faint band was seen and high level if a strong band was seen.

Semi-quantitative analysis was performed in a similar manner with the following genes and primers:

IFITM1, amplification at 62 °C.

Forward 5'-AGATGCACAAGGAGGAACATGAG-3'

Reverse 5'-ACTGTCACAGAGCCGAATACCA-3'

PIM1, amplification at 62 °C.

Forward 5'-CAAGGACGAAAACATCCTTATCGA-3'

Reverse 5'-ATCTTGCATCCATGGATGGTTC-3'

2.4.5 Screening for Chuvash polycythaemia

The most common mutation described in Chuvash polycythaemia is caused by a point mutation in exon 3 of the Von Hippel Lindau (VHL) resulting in the Arg200Trp substitution in the VHL protein. Screening for this mutation was performed using a direct restriction digest technique. A 19ul mix was made up consisting of: 2ul 10 x Bioline Reaction Buffer + 0.4ul 10mM dNTPs + 0.8ul 25mM $MgCl_2$ + 12.1ul sterile water + 1 ul each of forward and reverse primers (forward: 5'-CCTTGTACTGAGACCCTAGTC-3', reverse: 5'-AGGAAGGAACCAGTCCTGTATC-3') + 2ul Betaine + 0.1ul Biotaq DNA polymerase. Sample DNA was diluted 1:10 and 1ul added to the reaction mix. PCR was performed with 35 cycles of 95^0C for 30 seconds + 62^0C for 30 seconds + 72^0C for 30 seconds followed by termination at 72^0C for 5 minutes. Digestion was performed in a waterbath at 37°C for 4-16 hours with 5ul PCR product added to 0.5ul BsrB1 + 2ul 10xNEBuffer 2 (R0102S, New England Biolabs) + 3.5ul sterile water. Products were resolved on 1.5% agarose gel with added Ethidium Bromide and assessed as per figure a. Samples showing undigested bands were sequenced to confirm presence of the mutant.

2.4.6 Screening for V617F Jak 2 mutation

Screening was performed by PCR with a mismatched primer designed to introduce a restriction digest site for the Afl III enzyme into wild type Jak 2 products: An 18.5ul mix was made up consisting of 2ul 10 x Bioline Reaction Buffer + 0.4ul 10mM dNTPs + 0.8ul 25mM $MgCl_2$ + 13.3ul sterile water + 0.5 ul each of forward and

reverse primers (forward = 5'CAAGCATTTGGTTTTAAATTATGGAGTACGT-3', reverse = 5' TAAATTATAGTTTACACTGACACCTAG-3'). 18.5ul per sample was aliquoted out and a drop of mineral oil was overlaid. Sample DNA solution 0.5ul was added through oil and the samples were incubated at 95^0C for 5 mins then held at 85^0C for addition of 1ul Biotaq DNA Polymerase diluted 1:10 with sterile water. PCR was performed with 33 cycles of 95^0C for 30 seconds + 60^0C for 30 seconds + 72^0C for 30 seconds followed by extension at 72^0C for 10 minutes.

Overlying oil was removed and PCR products were then underwent digested: 8ul PCR product was added to 2ul sterile water + 0.5ul Afl III (R0541L, New England BioLabs) + 1ul 10x NEBuffer 3 + 0.5ul BSA (both New England Biolabs). Samples were incubated for 3-16 hours at 37^0C together with undigested controls. Products were then mixed with 3ul 6x Loading Dye (see 2.2.4) and loaded onto a 4% agarose gel with added Ethidium Bromide and concomitant known mutant and wild type controls.

2.4.7 Quantitative RQ-PCR by the SYBR Green technique

Reverse transcription was performed as per 2.4.3. Standard curves were created for all primers using the SYBR Green technique and compiled as described in 6.2.1. Reactions were performed in duplicate or triplicate with negative controls. cDNA (5ul) was added to a 20ul mixture of 6.5ul sterile water, 0.5ul each forward & reverse primers (IFITM1 forward 5'-CGACCATGTCGTCTGGTCCCTG-3', IFITM1 reverse 5'-CAGATGTTCAGGCACTTGGCGGTG-3', Pim1 forward 5'-CGAGCATGACGAAGAGATCAT-3', Pim1 reverse 5'-TCGAAGGTTGGCCTATCTGA-3', ABL forward 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3', ABL reverse 5'-GATGTAGTTGCTTGGGACCCA-3', Beta 2 microglobulin forward 5'-TCCTGAAGCTGACAGCATTCG-3', Beta 2 microglobulin reverse 5'-TCCATTCTCTGCTGGATGACG-3') & 12.5ul SYBR Green PCR Master Mix (4309155, Applied Biosystems). Reactions were performed using default settings on an ABI Prism 7700 and analysed using Sequence Detector v1.7 software (Applied

Biosystems). Average threshold cycle (Ct) was calculated and results plotted using the formula $2^{(\text{mean Ct housekeeper gene-mean Ct gene of interest})}$ and plotted on standard histograms.

2.5 Tumour cell lines

Tumour cell lines were recovered from in house samples stored in liquid nitrogen as per 2.2.1. They were routinely cultured at 37^0C 5% CO_2 in RPMI or IMDM with 10% added fetal calf serum and appropriate added cytokines [R10, I10].

2.5.1 Cytokine dependent cell lines

The following cytokine dependent cell lines were used:

TF1	human erythroleukaemia
BAF3	murine pro-B lymphoid line
Mo7e	human acute megakaryoblastic leukaemia
32D	murine myeloid line

Human cells were cultured in R10 + 20ng/ml GM-CSF. Murine lines were cultured in R10 + 10ng/ml mIL3 (Peprotech EC Ltd).

2.5.2 Cytokine independent cell lines

The following cytokine independent cell lines were used from culture in R10:

HEL	human erythroleukaemia
K-562	human chronic myeloid leukaemia in blast crisis
FDCP1*	murine bone marrow *stably expressing BCR-ABL (gift from R.Chopra, Paterson Institute of Cancer Research)

2.6 Western blotting

2.6.1 Starvation, Pre-inhibition & stimulation of cells

All cells were selected to give 10^6 cells per experimental point. Cytokine dependent cell lines were washed 3 times in 50ml PBS and resuspended at a density of 10^6/ml in fresh R10 overnight without added cytokines at 37^0C 5% CO_2. Primary cells were selected and washed in the same way but resuspended in IMDM with 5% BIT (serum-free medium, BIT 9500, Stem Cell Technologies containing bovine serum albumin, human recombinant insulin and human transferrin in IMDM) for 2-3 hours. Inhibitors were added at the desired concentration and samples were further incubated for 30-60 mins at 37^0C (+ 5% CO_2 if experimental period >2 hours). Stimulation was performed with the appropriate cytokine added for incubation periods of 10 mins to overnight.

The following inhibitors were used:

PI3 kinase inhibitors:

LY294002 (L-7962, LC Labs), PI103 (528100, Calbiochem), PI387 (Piramed Pharma, Slough UK), TGX-221 (10007349, Cayman Chemical Company), D030 (kind gift of Prof B. Vanhaesebroeck, Ludwig Institute for Cancer Research, London)

Jak 2 inhibitors:

Go6976 (365250, Calbiochem), Jak Inhibitor 1 (420097, Calbiochem)

PKC inhibitor:

Go6983 (365251, Calbiochem)

MAPK inhibitor:

U0126 (U-6770, LC Labs & V1121, Promega)

mTOR inhibitor:

Rapamycin (R5000-5M, LC Laboratories)

Pim-1 inhibitor:

LY294002

cKIT inhibitor:

Imatinib (Gleevec, Novartis Pharmaceuticals)

The following cytokines and stimulatory agents were used:

Erythropoietin, GM-CSF, Interferon alpha, rhIL3, IGF, mIL3, SCF (see 2.2.4, 2.3.3)
Thrombopoietin (gift from Amgen Inc, Seattle, USA)
TPA tetradecanoyl phorbol-myristate acetate (Calbiochem)

2.6.2 Protein lysates

At the designated time point cells were recovered, centrifuged at 2000 rpm/400g for 3 mins, washed in 1ml PBS then centrifuged at 2000 rpm for 3 mins again. The liquid phase was removed and the cell pellet suspended in 40ul ice cold lysis buffer and incubated on ice for 10-60 mins (Lysis buffer: Tris pH 7.5 50mM, NaCl 100mM, Triton X-100 1% [X-100-RS], DDW & serine protease cocktail 1:100 [P8340, Sigma-Aldrich]). Samples were then centrifuged at 14,000 rpm for 10 mins at 4^0C. The liquid phase was aspirated to a fresh tube and 10ul 5 x sample buffer added (5 x Sample buffer: 0.5M Sucrose, 15% Sodium dodecyl sulphate, 312.5mM Tris pH 6.9, 10mM EDTA, 0.05% Beta-mercaptoethanol & 0.0025% Bromophenol blue [Sigma-Aldrich]). Samples were boiled for 5 mins then stored at -20^0C pending electrophoresis.

2.6.3 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) & nitrocellulose transfer

Gels were prepared at 10%, 7.5% or 12.5% polyacrylamide concentration using the following components (DDW, 1M Tris pH 8.8 (pH 6.8 for stacking gel), ProtoGel (A2-0072, Geneflow Ltd), Sodium Dodecyl Sulfate (L4509, Sigma-Aldrich), Ammonium Persulfate (A3678, Sigma-Aldrich), TEMED (T9281, Sigma-Aldrich). The separating gel was prepared and poured into a Mighty-Small Hoefer gel caster (Hoefer scientific instruments). Isobutanol was layered on top and the gel was allowed to polymerise at room temperature. The isobutanol layer was then removed and rinsed off with DDW. A stacking gel was prepared, layered on top and a 10 or 20 well comb inserted. Once set the comb was removed and the wells rinsed to remove

unpolymerised acrylamide with 1 x Running buffer (10 x Running buffer: Tris 30.3g, Glycine 144.2g, SDS 10g & DDW 1 litre). The gels were then transferred to the running apparatus.

One sample well was loaded with pre-stained molecular size marker (S6-0028 ProtoMarkers, Geneflow Ltd) with equal volumes of sample lysate in each subsequent well (approximately 0.3 x 10^6 lysed cells per point). Gels were electrophoresed through 1x Running buffer using Hoefer gel apparatus at 150V until adequate separation had occurred. Gels were transferred using a semi-dry blotter (TE77 PWR, Amersham Biosciences) over 1 hour at a constant current of 0.8 amps with 1x Transfer buffer (10 x Transfer buffer: Tris 30.3g, Glycine 144.2g, Methanol 100ml & DDW 1 litre) onto Hybond-C Extra nitrocellulose membranes (RPN82E, GE Healthcare Life Sciences). Following transfer, membranes were blocked using 5% dried non-fat milk (Marvel) in PBST (PBS + 0.1% Tween-20 [P5927, Sigma-Aldrich]) for 1 hour then washed 3 times in PBST prior to incubation with primary antibodies.

2.6.4 Primary antibodies & incubation

Primary antibodies were made up according to manufacturers' instructions in PBST with 5% Bovine serum albumin (Sigma-Aldrich). Where phospho-specific antibodies were used membranes were incubated overnight at 4^0C. For other antibodies incubation was for 1-4 hours at room temperature.

The following Phospho-specific antibodies were used:

Phospho-Akt (Ser 473)	9271, Cell Signalling Technology
Phospho-Akt (Thr 308)	9275S, New England Biolabs
Phospho-Akt (Ser473)	44-621G, Biosource
Phospho GSK3 a/b (Ser 21/9)	9331S, Cell Signalling Technology
Phospho p44/42 (Thr202/Tyr204)	9211, Cell Signalling Technology
Phospho Stat 5 (Y694)	9351, Cell Signalling Technology
Phospho Jak 2 (Y1007/1008)	44-426G, Biosource
Phospho Stat 3 (Tyr 705)	9131, Cell Signalling Technology
Phospho Stat 1 (Tyr 701)	9171, Cell Signalling Technology

Phospho P70S6 kinase (Thr389)	9205, Cell Signalling Technology
Phospho S6 ribosomal protein (Ser 235/236)	2211S, Cell signalling Technology
Phospho RSK1 (Thr359/Ser363)	04-419, Upstate Biotechnology Inc

Other primary antibodies used:

Pim-1	sc-13513, Santa Cruz Biotechnology Inc
Alpha Tubulin	T-3526, Sigma
Stat 5b	C-17, Santa Cruz Biotechnology Inc
Total Akt	9272, Cell Signalling Technology
Erk 2	C-14, sc-154, Santa Cruz Biotechnology Inc
Beta-catenin	610154, BD Transduction Laboratories
S6 ribosomal protein	54D2, 2317, Cell Signalling Technology
P110 alpha	4254, Cell Signalling Technology
P110 beta	sc-603, Santa Cruz Biotechnology Inc
P110 gamma	4252, Cell Signalling Technology
P110 delta	gift of Prof B. Vanhaesebroeck, Ludwig Institute for Cancer Research, London

2.6.5 Secondary antibodies & chemiluminescent analysis

After incubation with primary antibody, membranes were washed 3 times in PBST, and incubated for 1 hour in 1:10,000 horseradish peroxidase conjugated anti-mouse or anti-rabbit immunoglobulin (NXA931, NA934V Amersham Biosciences UK Ltd) made up in PBST with 2% Marvel. After incubation with this secondary antibody, membranes were washed 3 times and protein bands detected by enhanced chemiluminescence (ECL Plus, RPN2132, GE Healthcare Life Sciences or EZ-ECL, 20-500-120 Geneflow Ltd) as per manufacturers instructions. The nitrocellulose membrane was then exposed to autoradiography film (Hyperfilm^{TM}, Amersham Life Sciences).

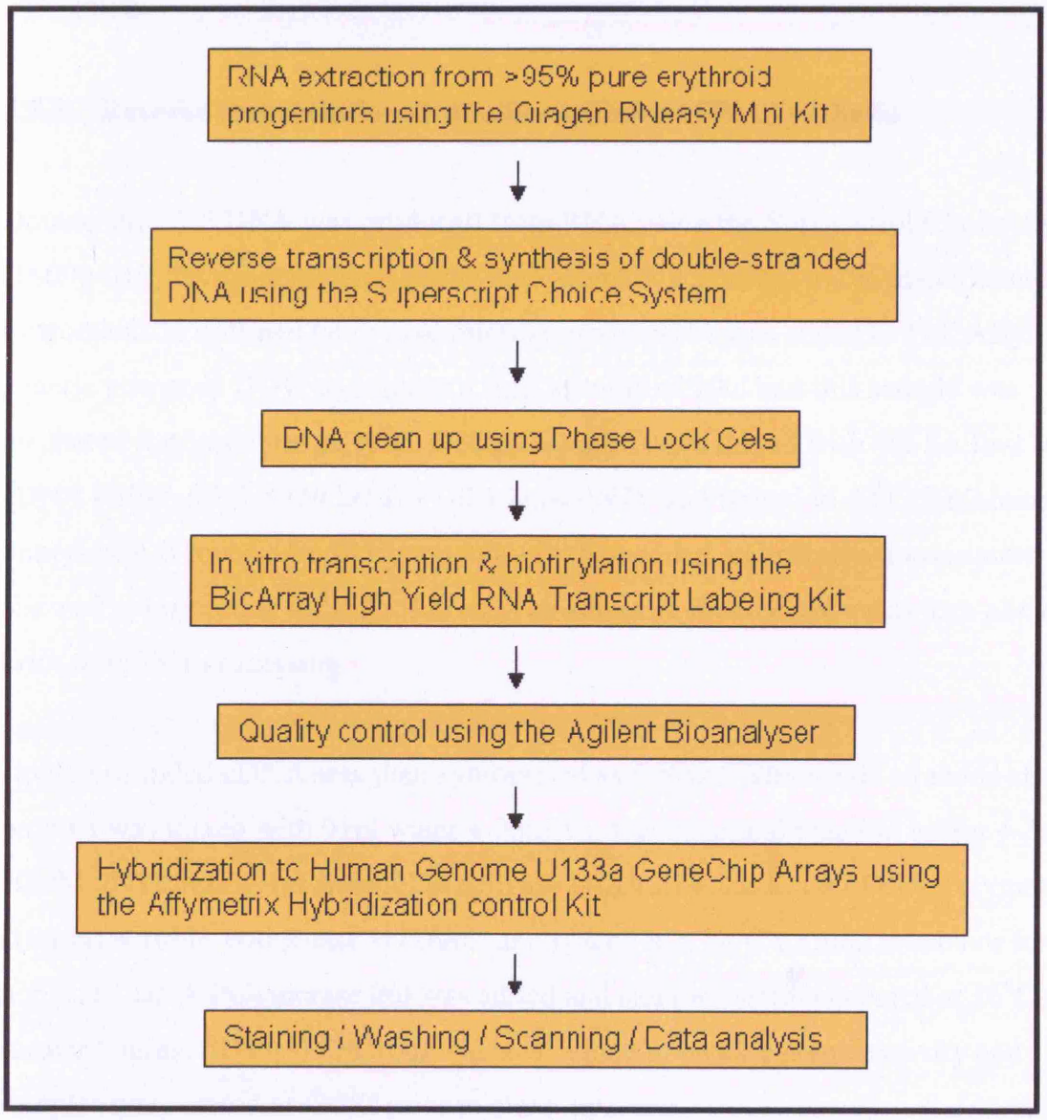
2.6.6 Densitometry

Exposed autoradiography films were scanned using an Epson Stylus Photo RX620 scanner and densitometry performed with ImageJ software for PCs downloaded from the NIH website (rsb.info.nih.gov/ij/download.html)

2.7 Microarrays

Gene expression profiling of erythroblasts was performed using Affymetrix U133a GeneChips according to the Affymetrix standard protocols represented in Figure 2[2] and detailed in the following sections. Fourteen GeneChips were performed in total. Of these 14, 6 were from patients with PV (2 homozygous, 4 heterozygous for V617F JAK2), 4 were from patients with IE and high / normal serum erythropoietin & 4 were from normal volunteers.

Figure 2[2]: Flow chart indicating processing steps to microarrays
(steps according to standard Affymetrix protocols for U133a GeneChips)



2.7.1 RNA extraction Qiagen method

RNA was extracted from Day 9 erythroid precursors where >1 x 10^6 cells of >95% purity as assessed by morphology and immunophenotyping had been produced. RNA was extracted using the RNeasy Mini Kit (74104, Qiagen) using sterile equipment and according to the manufacturers' instructions. Samples were centrifuged at 1300 rpm for 5 mins and washed with 1.5ml PBS. Cells were vigorously lysed in 350ul RLT buffer with added beta-mercaptoethanol and 70% ethanol was added for RNA precipitation. This solution was passed down an RNeasy mini column by brief centrifugation at 13,000 rpm and the column was washed with RW1 and RPE buffers. RNA was recovered into 30ul DDW in a clean tube. Quantity and quality were assessed by spectrophotometry using the Gene Quant pro (Amersham-Pharmacia) and suitable samples (A260/A280 = 1.9-2.1) were stored at -20^{0}C pending further processing.

2.7.2 Reverse transcription & double-stranded cDNA synthesis

Double stranded DNA was produced from RNA using the Superscript Choice System (18090-019, Invitrogen) using sterile equipment & according to the manufacturer's instructions as outlined here: Five micrograms of RNA was added to 1uL Affymetrix generic primer in DDW to achieve a final volume of 20ul and this sample was incubated for 5 minutes at 70^{0}C. Cooled samples were mixed with 4ul 5 x first strand cDNA buffer + 2ul 0.1M DTT + 1ul 10mM dNTP and heated to 42^{0}C for 2 mins. Superscript II reverse transcriptase 1ul was then added and reverse transcription was allowed to proceed at 42^{0}C for 1 hour. The single stranded cDNA was then cooled prior to further processing.

Double stranded cDNA was then synthesised as follows: The single stranded cDNA product was mixed with 91ul water + 30ul 5 x second strand reaction buffer + 3ul 10mM dNTP mix + 1ul E. coli DNA ligase (10U/ul) + 4ul E. coli DNA Polymerase I (10u/ul) + 1ul E. coli Rnase H (2u/ul) and placed in a 16^{0}C cooling incubator for 2 hours. T4 DNA Polymerase 2ul was added and samples were incubated at 16^{0}C for a further 5 mins. EDTA 0.5M 10ul was then added to block enzyme activity and samples were stored at -20^{0}C prior to clean-up.

Double-stranded cDNA was cleaned with use of Phase Lock Gels (Eppendorf): Double stranded cDNA was mixed with 162ul of 25:24:1 Phenol:Chloroform:Isoamyl alcohol (saturated with 10mM Tris-Hcl ph 8.0, 1mM EDTA). Mixed samples wre loaded onto Phase Lock Gels which had been pelleted at 12,000 rpm for 30 secs and subsequently centrifuged at 12,000 rpm for 2 mins. The residual aqueous upper phase was then transferred to a clean tube.

Ethanol precipitation of cDNA was performed by mixing in 0.5 volumes 7.5M NH_4OAc and 2.5 volumes absolute ethanol (stored at -20^{0}C) with 0.5ul Glycogen (for visualization of the pellet) and centrifugation at 12,000 rpm for 20 mins. The cDNA pellet was carefully washed twice with 80% ethanol (stored at -20^{0}C) with centrifugation at 12,000 rpm for 5 mins in between each wash. The air dried pellet of ds-cDNA was then suspended in 12ul RNase-free water and stored at -20^{0}C.

2.7.3 In vitro transcription of ds-cDNA, biotinylation & cRNA fragmentation

In vitro transcription (IVT) to produce biotinylated cRNA was performed using the BioArray High Yield RNA Transcript Labeling Kit (42655, Enzo) as per manufacturer's instructions. Five microlitres of ds-cDNA in solution was added to 4ul each of Tubes 1-4 + 2ul Tube 5 & 18ul DDW. Samples were mixed & incubated at 37^{0}C for 5 hours.

Half of the biotinylated IVT product was then purified using the Qiagen RNeasy Mini Kit: Twenty microlitres of biotinylated cRNA was mixed with 80ul sterile water and 350ul RLT buffer (containing 10ul fresh beta-mercaptoethanol / ml buffer) and 250ul 100% ethanol. The mixture was loaded onto a mini column and briefly centrifuged at 12,000 rpm. The column was washed twice with RPE buffer and the cRNA then eluted into 25ul DDW by centrifugation at 12,000 rpm for 1 minute. The quantity and quality of the product was checked by spectrophotometry as previously and quantity was adjusted for RNA input using the formula:

Adjusted cRNA = RNA post IVT – (RNA starting dose) x 5/24

Twenty micrograms of the biotinylated cRNA in solution was mixed with 2ul of 5 x fragmentation buffer for each 8ul of cRNA and made up to 40ul with sterile water. The mixture was incubated at 94^0C for 35 mins then stored at -20^0C after adequate fragmentation had been verified by running a small quantity of the product on an agarose gel.

2.7.4 Quality control procedures and microarray hybridisation

Once all 14 samples of fragmented biotinylated cRNA had been derived for the study, (6 patients with PV, 4 patients with IE and 4 normal control subjects), fragmented cRNA samples were sent to the MRC Gene Service for quality control (QC) checks and hybridisation. QC procedures were carried out using the Agilent bioanalyser. Three samples were subsequently prepared for hybridization using the Affymetrix hybridization control kit and hybridized to Test3 GeneChip arrays for 16 hours. Following hybridization the GeneChip arrays were stained and washed on the fluidics station and then scanned. At a later date all samples were prepared for hybridization using the Affymetrix hybridization control kit and hybridized to Human Genome U133a GeneChip arrays for 16 hours. Following hybridization the GeneChip arrays were stained and washed on the fluidics station and finally scanned.

U133a GeneChips were produced by Affymetrix following publication of the draft Human Genome data. With over 500,000 distinct oligonucleotide features and >22,000 probe sets a single array can be used to comprehensively assess gene expression including that of 14,500 well characterised human genes. The accurate statistical assessment of output data is complex given the large number of variables. Data from the 14 arrays studied in this project was supplied by the MRC Gene Service via web-based access directly to a University College London specialized biostatistician (Stephen Henderson) for analysis.

2.8 Proliferation and apoptosis

2.8.1 Cell viability as assessed by MTS

The MTS assay (CellTiter 96 Aqueous one Solution Cell Proliferation Assay, G3580, Promega) indirectly measures total viable cell number by evaluating dehydrogenase enzyme activity found in metabolically active cells. This assay was used to compare viable cell numbers between aliquots of identical cells (Day 9 erythroid precursors or specified cell lines) incubated under variable conditions to give a quantitative measure of relative cell survival.

The selected cells were washed 3 times in sterile PBS and resuspended in IMDM + 5% BIT or R10 at a density of 1-2 x 10^5/ml. Two hundred microlitre aliquots were added to a flat-bottomed 96 well tissue culture plate with one well containing medium only as a colorimetric baseline. Viable cell number under differing circumstances was evaluated by adding different concentrations of cytokines and / or inhibitors to each well with duplicates or triplicates of each experimental point. Plates were incubated at 37^0C, 5% CO_2 overnight or for longer where specified. MTS reagent (20ul) was then added to each well and colorimetry evaluated at 1-8 hours using Anthos 2001 (Anthos Labtec Instruments) with STINGRAY software. Derived data was exported into Excel. The mean of replicates was calculated after adjustment for background exposure (medium, no cells) and appropriate curves were constructed.

2.8.2 Apoptosis by Annexin V, Propidium Iodide and FACS

Cells were selected to allow 5 x 10^4 / experimental point. Cells were washed 3 times in sterile PBS and resuspended in 0.5ml IMDM + 5% BIT or R10 with or without added cytokines or inhibitors at 37^0C, 5% CO_2 for 6 hours to overnight.

When incubation was complete, a mixture of 200ul Annexin V buffer (10mM HEPES pH 7.4, 140mM $NaCl_2$, 5mM $CaCl_2$ + DDW) + 15ul Annexin V Fluos (1828681, Roche) + 15ul Propidium Iodide solution (P4864, Sigma-Aldrich) was made up and 10ul of this was added to each test sample. These samples were then incubated on ice

for 10 mins-1 hour. Flow cytometry was performed (Epics Elite, Beckman Coulter, High Wycombe, UK) to assess for the percentage of viable cells, early apoptotic cells (Annexin V positive only) and dead cells (Annexin V & PI positive).

CHAPTER 3 – RESULTS 1

Defining a cohort of patients with true or apparent polycythaemia & characterising normal and aberrant erythropoiesis in vitro

3.1 Defining a cohort of patients with elevated haematocrit

During the 3 years of the study several hundred new patients with an elevated haematocrit were referred to the the Polycythaemia Clinic at UCLH. I saw all new patients and continued the care of the patients already in the clinic. The information presented in this chapter is derived from these patients. All patients with a suspected diagnosis of Polycythaemia Vera (PV) underwent research analysis during the study period. Material from other patients and normal controls was collected for comparative purposes. Research findings were discussed with patients and entered into their clinical notes.

3.1.1 Clinical information and standard laboratory data

Full clinical data was available on 62 patients with true polycythaemia, 4 patients with pseudopolycythaemia (Ps) and compared with 10 normal individuals. Of the 62 patients with true polycythaemia, 27 met the Polycythaemia Vera Study Group (PVSG) diagnostic criteria for PV. [Pearson et al, 1996] Of the 35 remaining patients 11 were classified as having Secondary Polycythaemia (SP) with a recognized primary medical cause for a polycythaemic state. The 24 remaining patients were classified as having Idiopathic Erythrocytosis (IE). Some of the individuals with SP and IE appeared to spontaneously remit during the study period in that they became independent of venesection for haematocrit control. In the PV group 1 heavy smoker died of pneumonia aged 85 and 1 patient went on to develop Acute Myeloid Leukaemia.

Disease-specific drug therapy for all patients was based on clinical guidelines [McMullin et al, 2005] but adapted to individual circumstances and preferences.

Actual treatment is shown in Table 1[3]. Other diagnoses including hypertension (HT), elevated lipids (lipids), diabetes mellitus (DM) and gout are shown in Table 2[3].

Table 1[3]: Disease-specific drug therapy of the patient cohort

(numbers of patients shown, +HC = also on Hydroxycarbamide, in the Aspirin group* 1 patient ceased aspirin due to complications, ** 1 patient changed to clopidogrel due to complications with aspirin)

		Aspirin		Warfarin	
	Total	**alone**	**+HC**	**alone**	**+HC**
Polycythaemia Vera	27	8	5*	1	2
Idiopathic erythrocytosis & Secondary polycythaemia	35	11**	7	0	0
Pseudopolycythaemia	4	2	0	0	0

Table 2[3]: Other drug therapy of the patient cohort

(numbers of patients shown receiving drugs to treat the indicated conditions, HT = hypertension, Lipids = elevated lipids, DM = Diabetes Mellitus)

	Total	**HT**	**Lipids**	**DM**	**Gout**
Polycythaemia Vera	27	8	3	2	3
Idiopathic erythrocytosis & Secondary polcycythaemia	35	4	3	2	3
Pseudopolycythaemia	4	4	2	0	0

Of the 27 patients with PV, 3 had had an arterial vascular event and 4 a venous thrombosis. Of the 35 patients with IE/SP, 6 had had an arterial and 5 a venous event. Two of the pseudopolycythaemics had had an arterial event, none had had a venous event. Two in each of the PV and IE/SP groups had an unconjugated hyperbilirubinaemia. Three patients in the PV group and 1 in the IE/SP group had a

family history of polycythaemia or thrombocythaemia. One patient in each of these groups had a family history of venous thromboembolism.

Of the 27 patients with PV, 13 had splenomegaly, either diagnosed on abdominal ultrasound scan, or mildly-moderately clinically palpable. In the IE/SP group 1 patient had mild splenomegaly and 1 had had a splenectomy for massive splenomegaly following a portal vein thrombosis in pregnancy (this patient was subsequently shown to be a compound heterozygote for the G20210A Prothrombin gene mutation & for Protein S deficiency).

Routine laboratory investigations are shown in Table 3[3]: Only high white cell count (WCC) and platelet count (Plt) were predictive of PV in this cohort. Serum Erythropoietin was only available for 9 patients with PV & was subnormal in 4 and low normal in 5. One patient who had borderline polycythaemia (red cell mass elevated but not at threshold) subsequently developed PV and went from a normal to a subnormal serum erythropoietin. In the IE/SP group 23 had normal, 4 had high and none had subnormal serum Erythropoietin.

Table 3[3]: Routine laboratory investigations
(numbers of patients shown, High B12 = serum B12 above the upper end of the normal range, Urate = serum urate, WCC = white cell count, Plt = platelet count)

	Total	**High B12**	**High Urate**	**High WCC**	**High Plt**
Polycythaemia Vera	27	2	3	17	10
Idiopathic erythrocytosis & Secondary polycythaemia	35	5	4	2	0

3.1.2 Erythropoietin independent colonies (EECs)

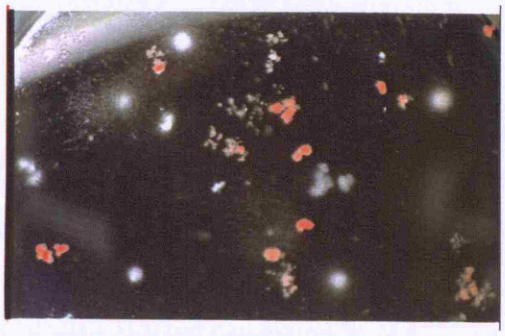
Erythroid colonies grown in the presence of erythropoietin are easily identifiable by light microscopy. The erythropoietin-independent colonies which are characteristic of PV are much harder to identify as shown in Figure 1[3] as they are considerably less numerous, smaller and less well haemoglobinized than their erythropoietin-replete counterparts. Where there was any doubt as to the presence or absence of EECs, culture plates were reviewed by an independent assessor and/or repeated on subsequent peripheral blood samples if possible.

Of the 27 patients with PV 25/27 (93%) had positive erythropoietin independent colonies (1 equivocal, 1 not done). Of the 35 patients with IE or SP none had positive EECs. Fourteen control subjects including 10 normal individuals had negative EECs. In 27 experiments where EECs were positive, the mean total numbers of erythroid colonies formed were 10 (no Epo) and 54 (Epo 2iu/ml). Thus EECs represented 19% of erythroid colonies in subjects with PV.

Figure 1[3]: Erythroid colonies from a patient with PV in the presence and absence of erythropoietin

(tissue culture plates showing both erythroid & non-erythroid colonies. Erythropoietin independent colonies are small and less well haemoglobinized as indicated within the red circles in b)

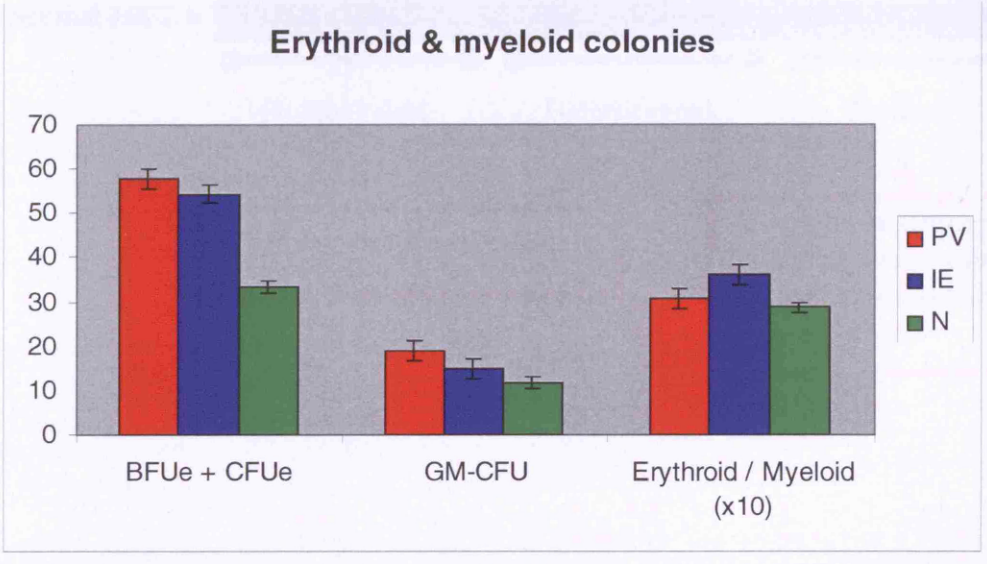
a] added erythropoietin



b] no erythropoietin (EECs)

In order to confirm that the presence of EECs in PV was not just due to an increased relative proportion of committed erythroid progenitors in mononuclear cell input, data from 80 EEC assays (including 33 PV, 37 IE & 10 normals) looking at maximal added erythropoietin only, was analysed. The mean number of erythroid colonies (BFUe & CFUe) and myeloid colonies (GM-CFU) was calculated and the results of this calculation are portrayed in Figure 2[3].

Figure 2[3]: The mean number of erythroid and myeloid colonies & the erythroid : myeloid ratio from 80 assays (33 PV, 37 IE & 10 normals)
($1x10^5$/ml mononuclear cells derived from peripheral blood were put into semi-solid medium with erythropoietin 2iu/ml, erythroid [BFUe & CFUe] and myeloid [GM-CFU] colonies were counted at Day 14 and mean values by diagnosis [PV = polycythaemia vera, IE = idiopathic erythrocytosis, N = normals] are shown here together with the erythroid : myeloid ratio)



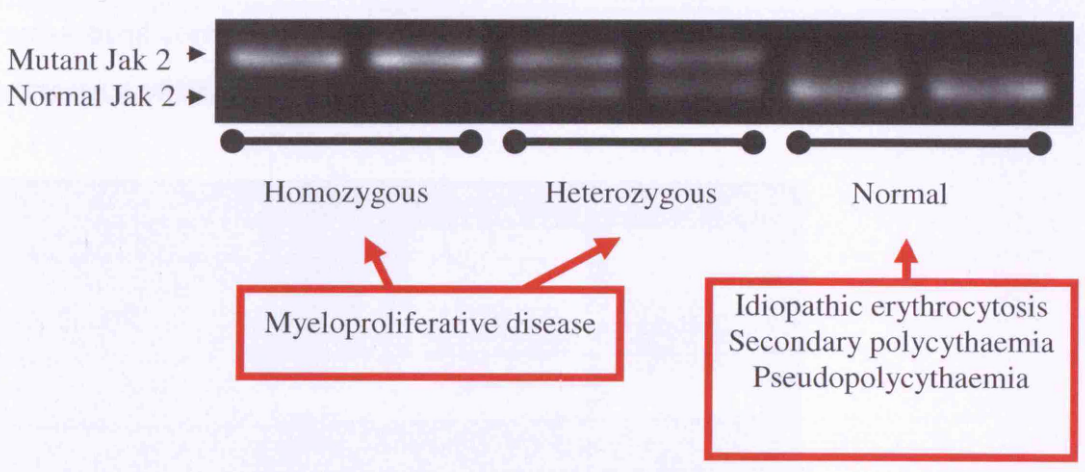
This data shows that significantly more erythroid colonies were formed from the PV and IE patients than the normal subjects but that there was no difference in erythroid:myeloid colony ratio between these groups. This confirms that whilst erythroid colony formation is increased in the PV and IE groups, it is not an excess of erythroid progenitors in PV that induces the formation of the EECs seen in this disease.

3.1.3 V617F JAK2 screening

Screening for V617F JAK2 was performed as described in 2.4.6 by PCR of neutrophil-derived DNA using a mismatched primer which introduced a cutting site into wild type JAK2 for the restriction enzyme Afl III. Products were resolved by agarose gel electrophoresis and representative samples are shown in Figure 3[3]. Of the 27 patients with PV, 25/27 (93%) expressed V617F JAK2 with 5 (20%) showing homozygous expression of the mutant form. All of the 35 patients with IE or SP & the 14 control subjects were wild type for Jak 2. Patients expressing V617F JAK2 are designated as 'Myeloproliferative disease' as the mutation is not specific for PV.

Figure 3[3]: PCR products for wild type and V617F mutant Jak 2

(homozygous / heterozygous mutant = V617F JAK2, normal = wild type JAK2)

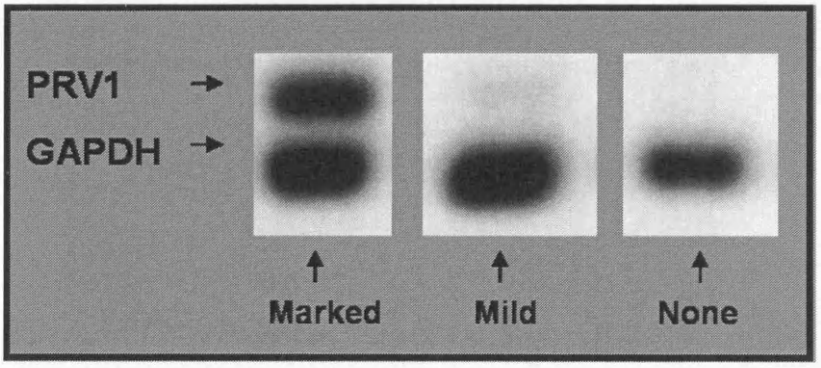


3.1.4 PRV1 mRNA expression

Detection of upregulated PRV1 mRNA expression was assessed semi-quantitatively by reverse transcription of neutrophil-derived RNA followed by simultaneous PCR of PRV1 and GAPDH as described in 2.4.4. Results were categorized into 3 groups with marked, mild or no upregulation of PRV1 as shown in the examples of Figure 4[3].

Of the 27 patients with PV 24/27 (89%) had marked upregulation of PRV1 mRNA. Of the 3 remaining patients with PV, 2 had mild upregulation of PRV1 and 1 patient was not assessed for PRV1 expression. None of the 35 patients with IE or SP had marked upregulation of PRV1 expression but 6 had mild upregulation .Of the 14 control subjects, only 1 had mild upregulation of PRV1.

Figure 4[3]: Semi-quantitative evaluation of PRV1 mRNA upregulation
(upper band corresponds to PRV1 product, lower band to GAPDH product, increased expression of PRV1 described as marked, mild or none)



3.1.5 Screening for Chuvash Polycythaemia

Sixty seven patient samples including 26 patients with PV were screened for the Arg200Trp VHL mutation described in hereditary Chuvash Polycythaemia. Screening for the mutation was performed as described in 2.4.5 by PCR of neutrophil-derived DNA. Use of the BSRB1 restriction enzyme with this methodology resulted in digestion of the wild type but not the mutant. Agarose electrophoresis of representative products is shown in Figure 5[3]. One of these 67 samples, derived from a patient with unmutated V617F JAK2 and polycythaemia secondary to carcinoma of the larynx, showed heterozygosity for the Chuvash mutation which was confirmed by sequencing as shown in Figure 6[3]. A further sample from a patient with new onset PV screened positive for the mutation but sequencing of the VHL gene showed that this was a false positive.

Figure 5[3]: Mutational screening for Chuvash Polycythaemia

(PCR products resolved by agarose electrophoresis, HET = heterozygous for ARg200Trp VHL, HOM = undigested sample, normal = wild type)

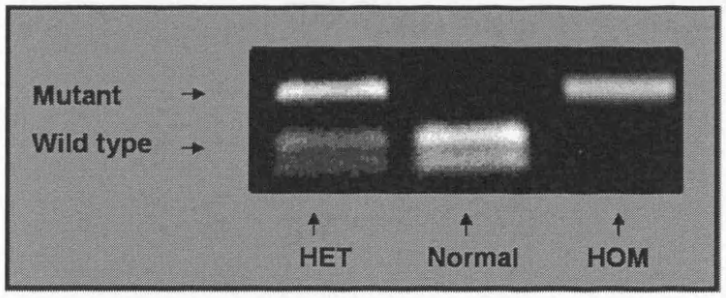
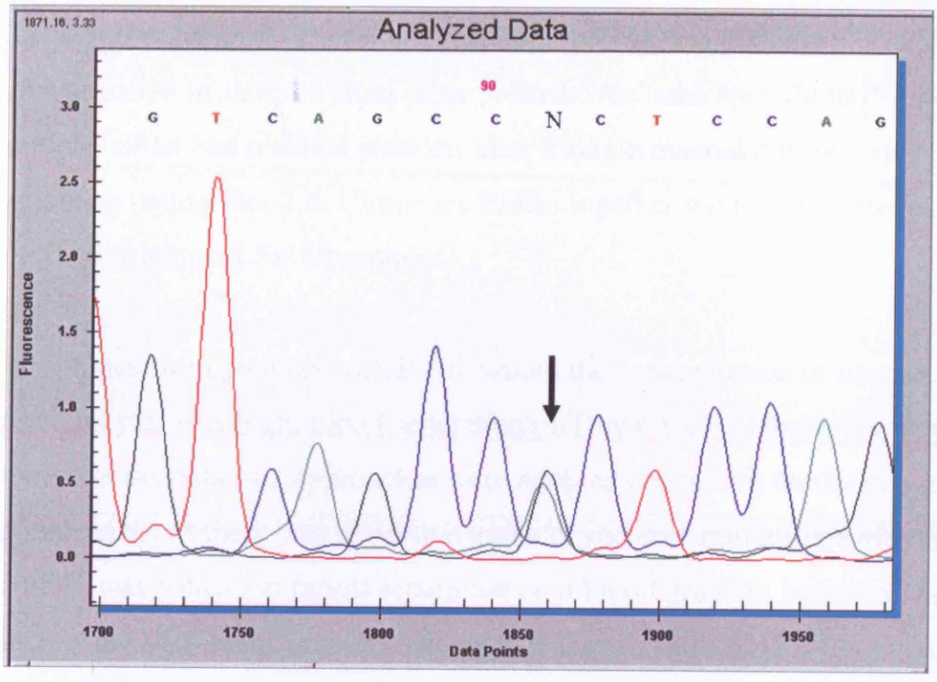


Figure 6[3]: Sequencing plot from a patient heterozygous for Chuvash Polycythaemia

(arrow shows heterozygous expression at the mutation site due to a single point mutation which results in substitution of arginine for tryptophan at position 200 of the VHL protein)



3.2 Liquid culture of erythroblasts

3.2.1 Optimisation of methodology

Throughout the study some patient samples had to be discarded due to technical sampling issues with venesection as described in 2.1.3 which led to clot formation in the venesection pack. This was a particular problem in patients with PV although it also occurred in samples from other patients. An issue specific to PV was that samples often had residual platelets after a single manual removal step so a 2-step approach (using Ficoll & Clinimacs Buffer together with slow centrifugation) was ultimately adopted for all samples.

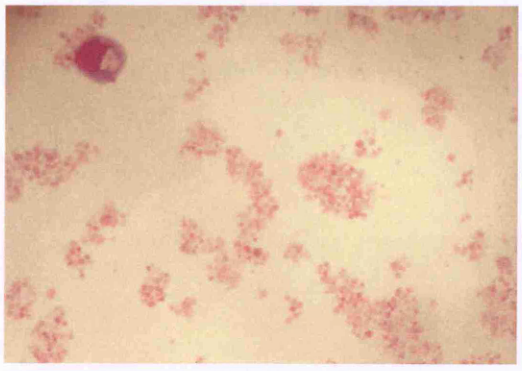
In samples from patients with PV in whom the concentration of immature circulating granulocytes was high, Ficoll separation on Day 0 was sometimes suboptimal. In these circumstances 2 approaches were adopted depending on the microscopic appearances: if there was excessive granulocytic contamination early on, the liquid culture was subject to repeat separation on a Ficoll gradient between Days 0-6. If there were still viable granulocytic cells at Day 6, anti-CD15 – conjugated microbeads were introduced into the negative selection cocktail described in 2.2.2 as per manufacturer's instructions (130-046-601, Miltenyi Biotec).

At the start of the study methodology was adapted to optimise the cellular yield of the protocol but minimize contamination with non-erythroid cells. A positive selection strategy from the outset initially seemed more attractive. Unfortunately an early positive selection approach using CD34 selection 'up front' on patient samples ultimately gave low numbers of erythroblasts with up to 20% contamination with non-erythroid myelomonocytic precursors at the end of the culture period. This was not a problem when large numbers of CD34+ mobilized cells were used but was a limiting factor for therapeutic venesection products containing low numbers of progenitor cells. A negative selection strategy 'up front' likewise gave limited cellular yields from patient packs.

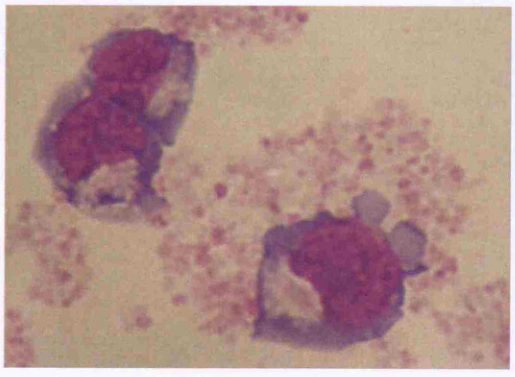
Attempts at a delayed positive selection strategy were confounded by the poor specificity of any single marker for early erythroblasts: CD36 is considered a valuable erythroid marker but when mononuclear cells at Day 6 of culture were selected for CD36 there was heavy platelet contamination as this marker is also expressed on platelets. Most of the erythroblasts were CD36+, however, a significant number of erythroblasts were lost in the CD36- fraction as shown in Figure 7[3] [a & b]. Likewise, the transferrin receptor, CD71, is known to be strongly expressed on erythroblasts but positive selection for CD71 gave a similar problem with macrophages as shown in Figure 7[3] [c & d].

Figure 7[3]: Cytospins of CD36 and CD71 selected mononuclear culture
(CD36 *positive* fraction is heavily contaminated with platelets, some erythroblasts were lost in the CD36 *negative* fraction [a & b], CD71 *positive* fraction is heavily contaminated with macrophages and numerous erythroblasts were lost in the CD71 *negative* fraction [c & d]

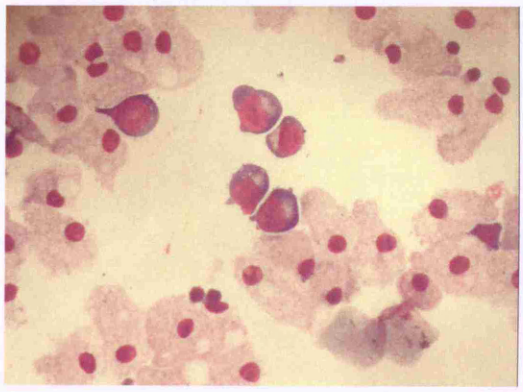
a] CD36 positive



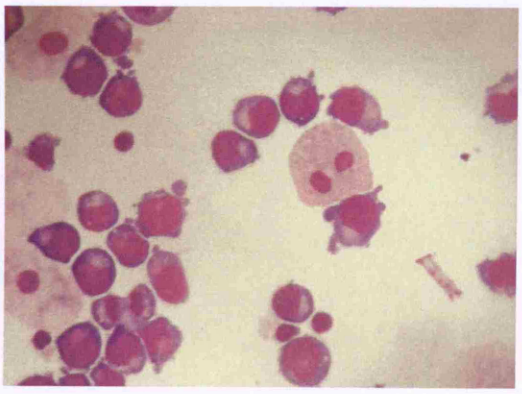
b] CD36 negative



c] CD71 positive



d] CD71 negative



In some samples (mainly from patients with PV) there was persistent macrophage contamination after negative selection at Day 6 which could not be corrected by immediate reselection. Residual macrophages were removed in these circumstances by 2 methods on Day 7/8: Negative selection using anti-CD14-conjugated microbeads only or adherence depletion. Adherence depletion was achieved by laying the tissue culture flask on its side for 1 hour and then aspirating non-adherent cells to a clean flask. This was then repeated as necessary.

One normal individual with an exacerbation of asthma had excessive eosinophil contamination at Day 9 and the sample had to be discarded. Following this, more careful attention was paid to take normal samples from volunteers who were completely well. Despite the high initial eosinophil and basophil counts in peripheral blood of some patients with PV, contamination of Day 9 cells with these cell types was <2% in PV patients.

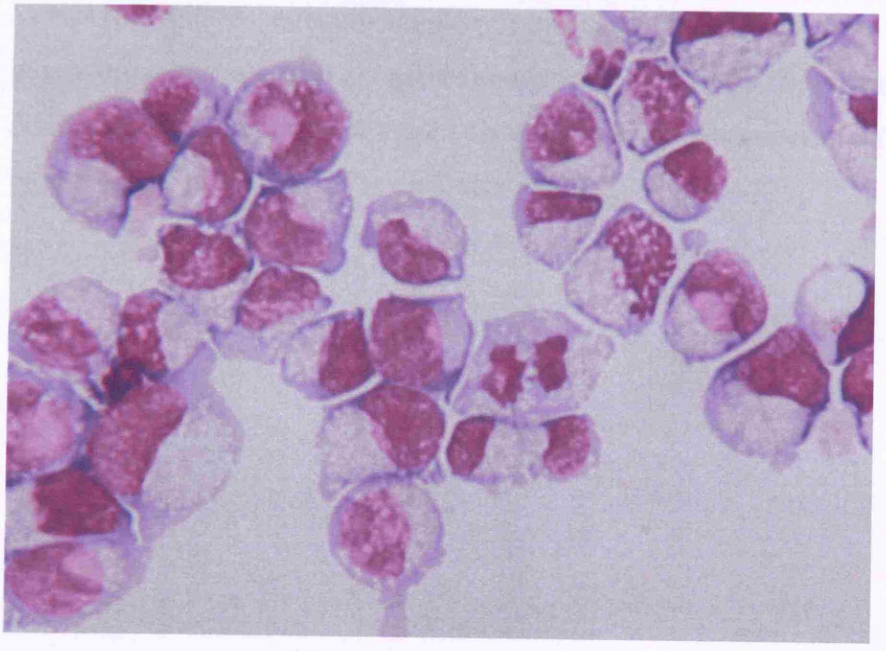
There were intermittent problems with infected cultures in the laboratory. Samples which showed evidence of infective contamination were immediately discarded. No antibiotics were added to the primary culture systems as it was felt this might prejudice interpretation of results.

3.2.2 Erythroblast morphology

All samples were morphologically assessed at Day 9 (or sooner if indicated by non-homogeneity of the cellular culture on microscopy). Some patients showed dysplastic features in derived basophilic erythroblasts but overall there was no significant morphological difference between patient samples. A representative cytospin is shown in Figure 8[3].

Figure 8[3]: The morphological appearance of Day 9 erythroblasts after cytospin and staining with MGG

(May-Grumwald-Giemsa staining shows characteristic basophilic erythroblasts with evidence of active mitosis, cytoplasmic vacuolation was an in vitro artefact noted in all experimental samples)



3.2.3 Erythroblast immunophenotype as assessed by flow cytometry

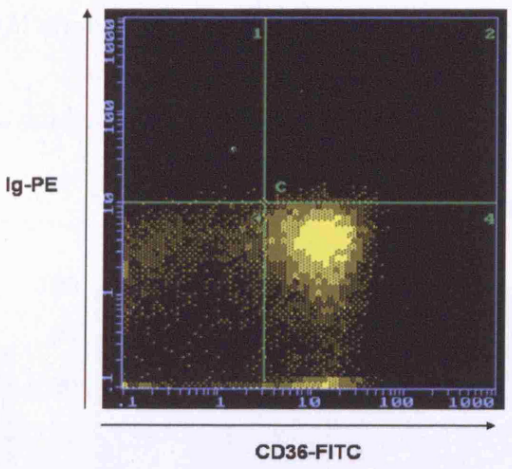
By Day 9 the viable cellular population was >90% erythroblasts by morphology (>95% for microarrays & 80-90% for erythroblasts derived from CD34+ progenitors). Figure 8[3] shows representative plots from one sample. The majority of erythroblasts were CD36+ with low/intermediate mean fluorescence intensity. CD71 was expressed in nearly all erythroblasts with high mean fluorescence intensity. CD117 was weakly expressed in a minority of erythroblasts. Glycophorin A was expressed at an intermediate level in approximately half of the cells. However, there was a spectrum of expression for this antigen.

Forty-three separate samples of day 8-10 erythroblasts were immunophenotyped. These 43 included 21 with PV (6 homozygous, 15 heterozygous V617F JAK2), 14 with IE, & 8 normal controls. Mean percentage positivity for each marker was calculated and these results are shown in Figure 10[3]. Similar results were noted when data was assessed by mean fluorescence intensity: There was no significant difference in immunophenotype between erythroblasts generated from peripheral blood either by diagnosis or by JAK2 status.

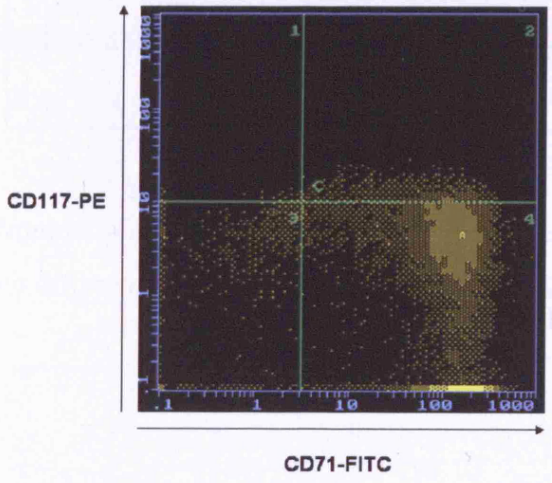
Figure 9[3]: Flow cytometric plots of Day 9 erythroblasts from a patient with IE after incubation with a] CD36-FITC + Ig-PE, b] CD71-FITC + CD117 PE, c] Ig-FITC + Glycophorin A-PE

(FITC conjugated antibodies [X axis] and PE conjugated antibodies [Y axis])

a]



b]



c]

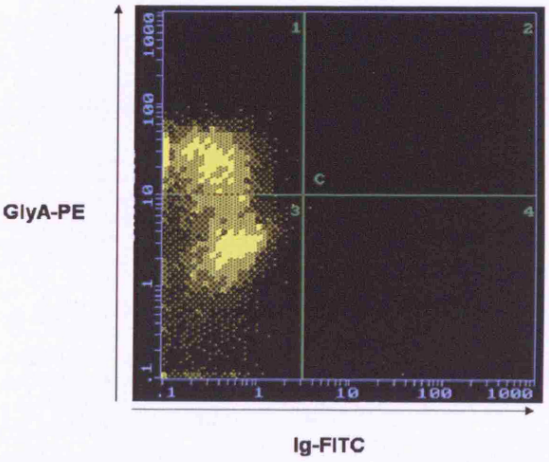
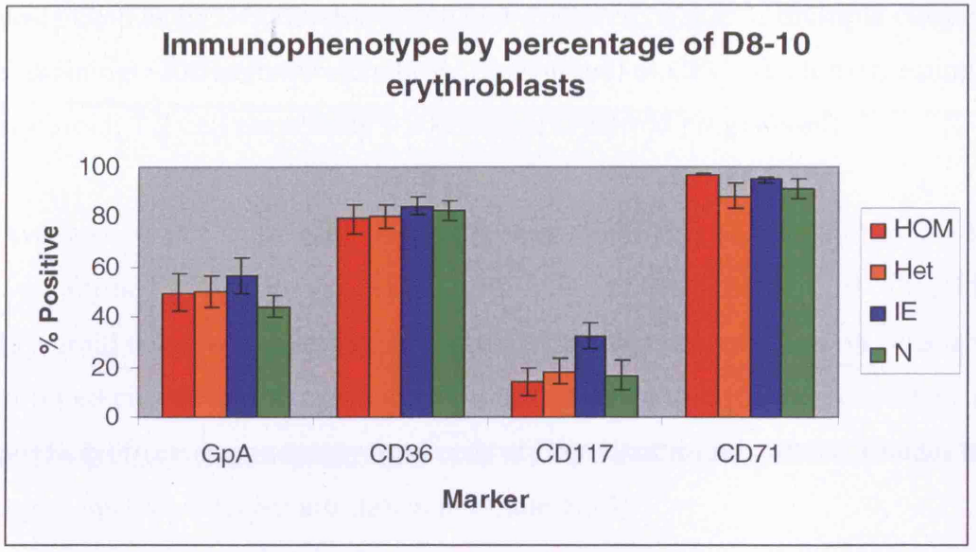


Figure 10[3]: Flow cytometric findings from 43 separate patient-derived samples according to diagnosis

(includes 6 patients with PV homozygous for V617F JAK2 [HOM], 15 patients with PV heterozygous for V617F JAK2 [Het], 15 patients with IE & 8 normal samples all wild type for JAK2, percentage positivity for GpA [Glycophorin A], CD36, CD117 & CD71 shown with calculated standard error of the mean)



Immunophenotyping of Day 13 cells in 7 samples (2 PV, 2IE, 3 normals) also showed no difference between PV and controls (data not shown).

3.2.4 Erythroid colony-forming potential of basophilic erythroblasts

In order to establish the erythroid colony-forming potential of the cultured cells, mononuclear cells derived from 6 normal peripheral blood samples were put into standard colony assays on days 0, 1, 2, 3, 4, 6, 8, 9 & 10 of in vitro liquid culture ($1x10^5$/ml pre-selection & $1x10^4$/ml post Day 6 negative selection). Erythroid colonies were enumerated after a further 14 days of culture in semi-solid medium and designated as BFUe (Burst-forming unit-erythroid; single or multiple clusters containing >200 immature erythroid progenitors) or CFUe (Colony-forming unit-erythroid; 1-2 cell clusters of 8-200 mature erythroid progenitors).

Averaged results from these assays are shown in Figure 11[3]: Erythroid colonies were formed from assays set up on cells up to and including day 10 of liquid culture. Erythroid colonies were predominantly BFUe from assays set up on cells up to Day 4 of liquid culture and increasingly CFUe thereafter although occasional BFUe were produced from assays set up from cells at Day 10 of liquid culture. Images of representative colonies are shown in Figure 12[3].

Figure 11[3]: The relationship between BFUe & CFUe according to the number of days of liquid culture

(Mean number of colonies derived from cells taken from liquid culture on the specified days and plated into semi-solid colony assays, BFUe = Burst forming unit erythroid, CFUe = Colony forming unit erythroid)

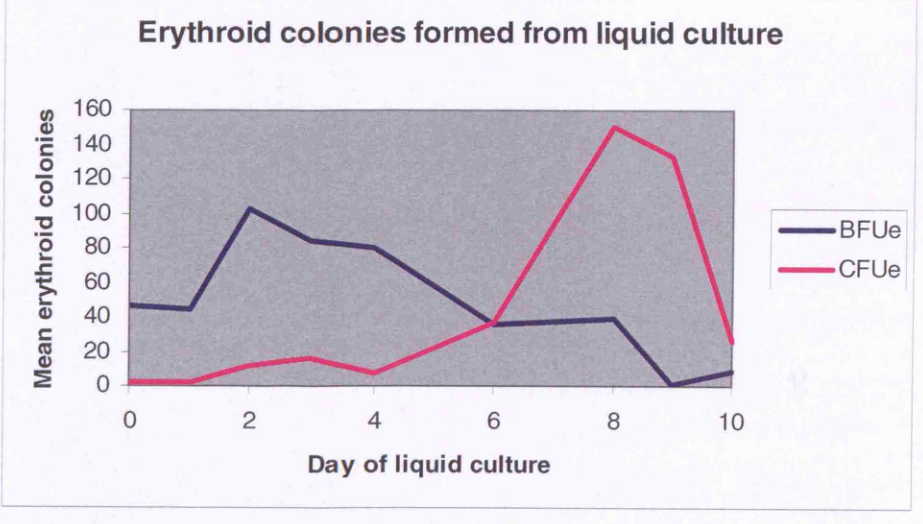
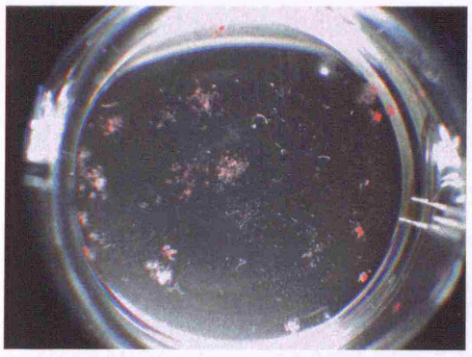
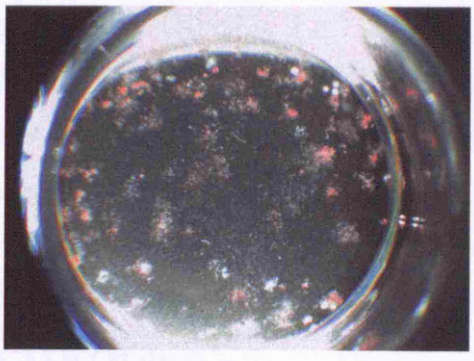


Figure 12[3]: Erythroid colonies from mononuclear cells in liquid culture
(images of colony plates after 14 days of semi-solid culture taken from liquid culture on the specified days)

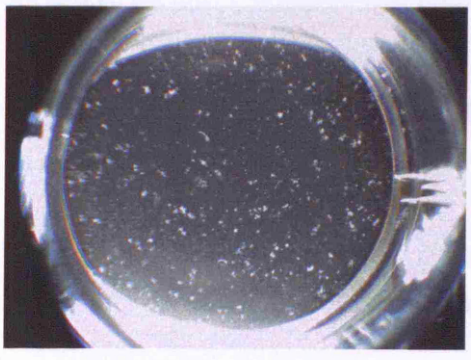
a) Day 0



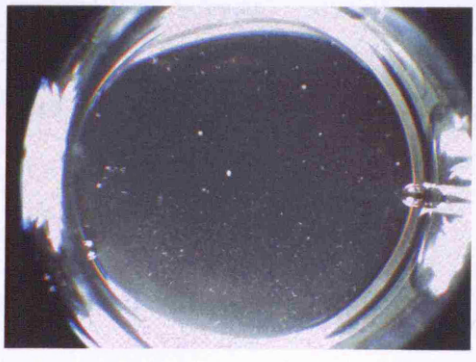
b) Day 4



c) Day 6



d) Day 9

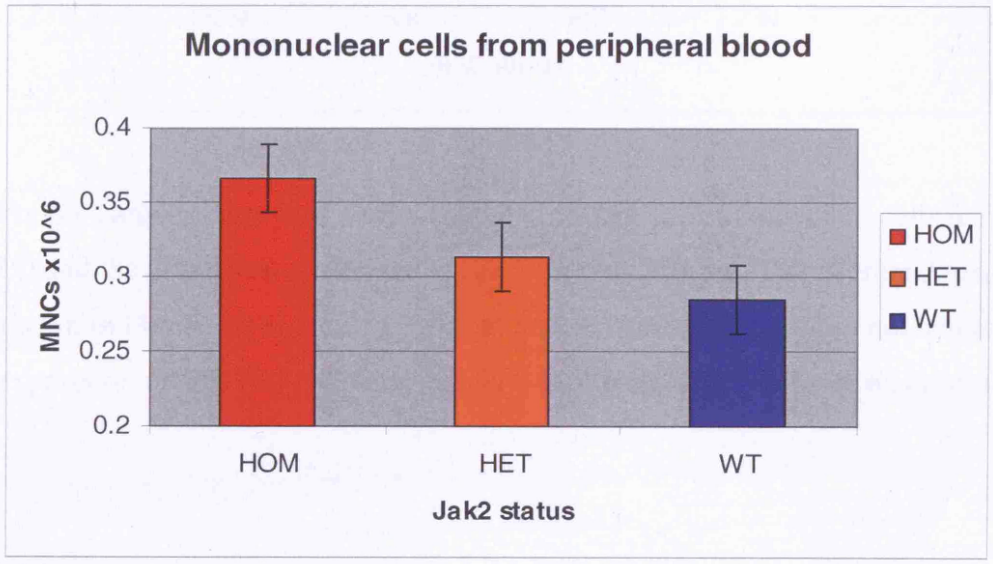


3.2.5 Proliferation profiles

Anticoagulated peripheral blood from 134 samples (13 homozygous for V617F JAK2, 38 heterozygous for V617F JAK2, 83 wild type for JAK2 including 52 from patients with IE/SP, 7 from patients with Pseudopolycythaemia & 24 from normal controls) was separated to obtain mononuclear cells as described in 2.2.2. The cellular yields per ml of anticoagulated blood are shown in Figure 13[3] and suggest a trend towards higher cell number according to V617F level. Median values x 10^6 for cellular yields / ml were 0.28 (homozygous), 0.23 (heterozygous) and 0.16 (wild type).

Figure 13[3]: The number of mononuclear cells (x10^6) per ml derived from anticoagulated peripheral blood by JAK2 status

(the mean number of mononuclear cells harvested per ml of venesected blood is shown & includes samples from individuals with PV expressing homozygous V617F JAK2 [HOM], heterozygous V617F JAK2 [HET] and samples from patients with IE/SP or normal controls expressing wild type JAK2 [WT], calculated standard error of the mean shown)

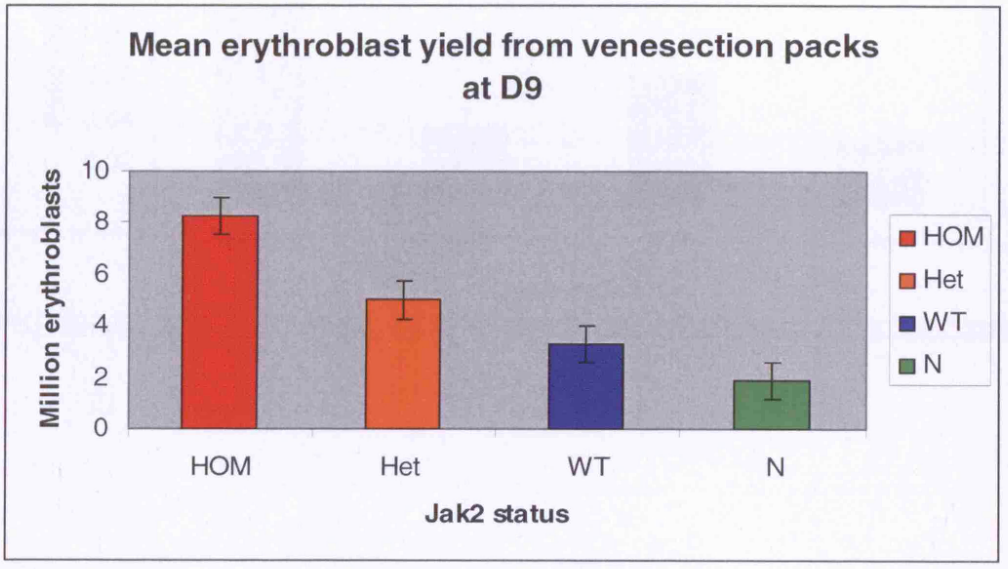


Overall, a higher number of erythroblasts were obtained after in vitro culture using samples from patients with PV compared with patients with IE/SP or from normal controls although there was considerable variation between individuals. Erythroblast counts from 81 venesection packs were available at Day 9 of in vitro culture (44 PV;

8 Homozygous V617F JAK2, 36 Heterozygous V617F JAK2, 30 IE/SP & 7 Normal subjects all expressing wild type JAK2). Values have not been corrected for pack volume or quality. Results are shown in Figure 14[3].

Figure 14[3]: Erythroblast yields from 81 venesection packs after 9 days of in vitro culture according to JAK2 status

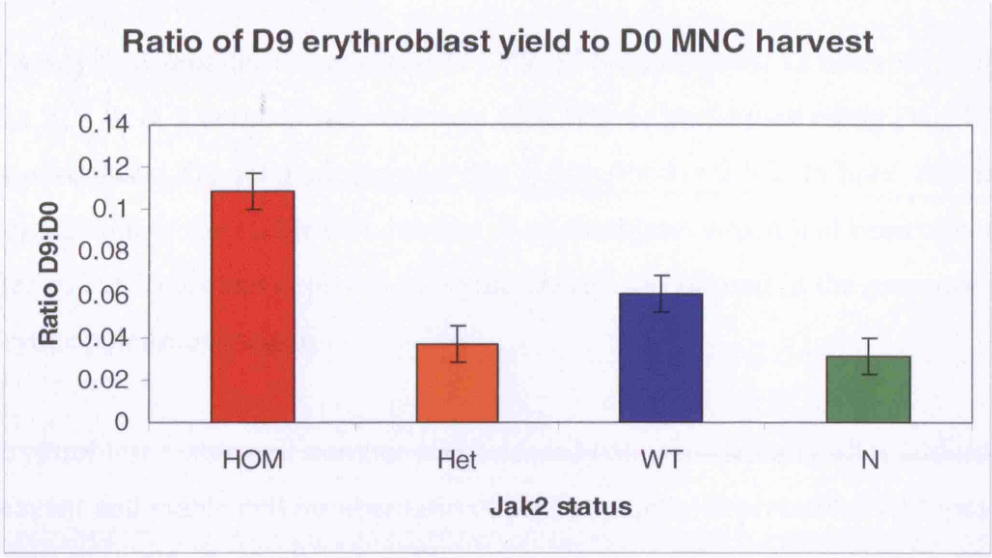
(number in millions of erythroblasts at D9 including 44 PV with V617F JAK2 [8 HOM = homozygous, 36 Het = heterozygous, 30 IE/SP [WT = wild type] & 7 normals [N], calculated standard error of the mean shown]



For 59 samples there was numerical data for both the mononuclear cells harvested on D0 and the erythroblasts derived at D9 of in vitro culture. The averaged results are shown in Figure 15[3] and suggest that homozygous, but possibly not heterozygous, expression of V617F JAK2 may confer a proliferation advantage over normal controls.

Figure 15[3]: The proportion of mononuclear cells which went on to form erythroblasts

(ratio of the numbers of D9 to D0 cells shown from 59 subjects including 29 with PV & V617F JAK2 expression [6 homozygous], 25 subjects with IE/SP & 5 normals [all wild type for JAK2], calculated standard error of the mean shown)



3.2.6 Resistance of PV erythroblasts to erythropoietin deprivation

It is well established that a proportion of circulating progenitor cells in patients with PV have potential to form erythroid colonies in the absence of erythropoietin (EECs). There is currently no data in the scientific literature to show whether resistance to factor deprivation is a feature of maturing erythroblasts in patients with PV.

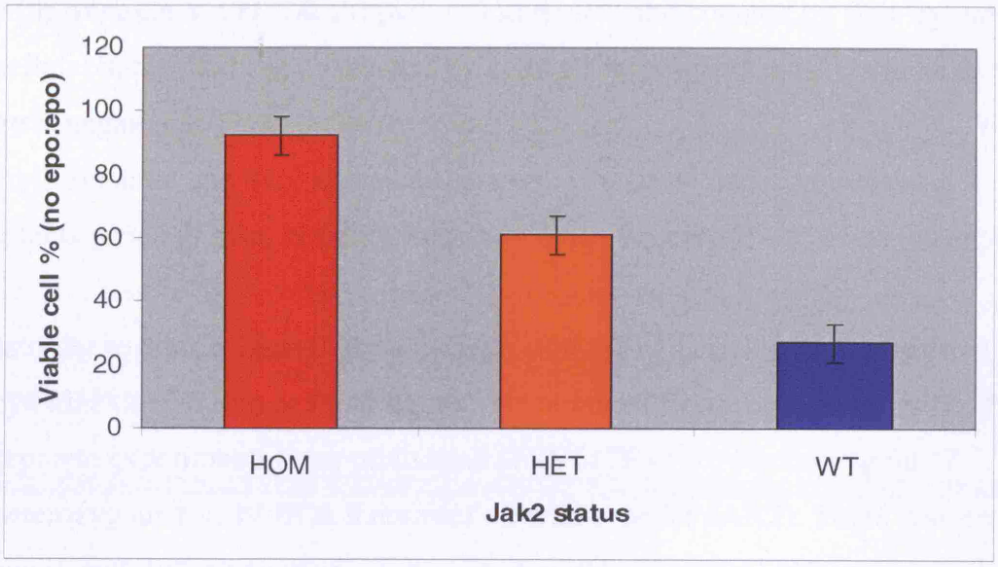
Twenty four separate experiments (14 PV, [2 homozygous, 12 heterozygous V617F Jak 2] 8 IE & 2 normals [all wild type JAK2] were performed using Day 9 erythroblasts. The methodology for this is described in 2.8.2. In brief, this involved comparison of the viable cell number of erythroblasts which had been either washed free of cytokines and deprived of erythropoietin or cultured in the presence of 2iu/ml erythropoietin overnight.

Erythroblast viable cell number was assessed colourimetrically after addition of MTS reagent and viable cell number ratio of erythropoietin-deprived / erythropoietin-replete samples was calculated. The averaged results from these experiments are shown in Figure 16[3].

These findings clearly illustrate that maturing erythroblasts from patients with PV are relatively resistant to factor deprivation and that this resistance may be greater where expression of V617F JAK2 is homozygous.

Figure 16[3]: Relative erythroblast viable cell number with erythropoietin deprivation as assessed by MTS assay in 24 separate experiments

(the ratio of viable cells without erythropoietin to viable cells with erythropoietin is shown, samples include 14 patients with PV; 2 homozygous [HOM] & 12 heterozygous [HET] for V617F JAK2 and 10 samples from subjects expressing wild type JAK2 [WT], calculated standard error of the mean is plotted)



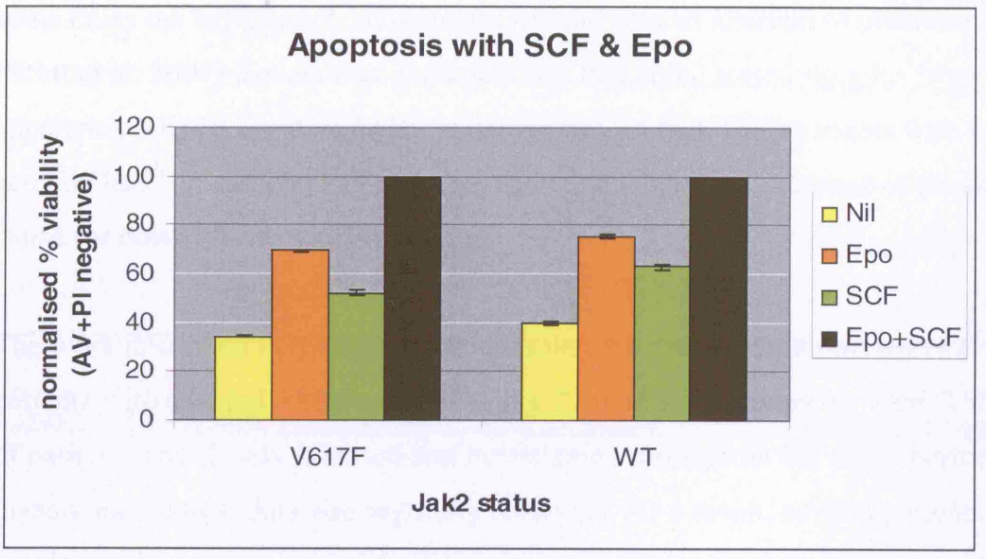
3.2.7 Apoptosis as assessed by Annexin V, Propidium Iodide & FACS

Nine preliminary experiments (2 PV, 3 IE, 4 normals) were performed to assess resistance to apoptosis with a range of doses of erythropoietin and or SCF. The methodology for this is described in 2.8.3. In brief, D9 erythroblasts were washed to remove residual cytokines and incubated overnight with or without added erythropoietin and/or SCF at a range of doses. The following day cells were stained with Annexin V-FITC & Propidium Iodide (PI) and counted by flow cytometry after cellular debris had been excluded by gating. The percentage of viable cells which were negative for both Annexin V and PI was calculated for each sample. Both erythropoietin and SCF prevented apoptosis in a dose dependent fashion & their effects were additive. No difference was noted between PV & control samples.

In order to confirm this finding, a larger number of samples were evaluated with cytokine deprivation or fixed high levels of added Erythropoietin or SCF: Thirty four separate experiments were performed (11 V617F+ PV; 4 homozygous, 7 heterozygous for, 14 IE & 9 normals all wild type for JAK2). There was no significant difference in apoptosis between PV and control cells which had been deprived of erythropoietin or SCF (see Figure 17[3]).

Figure 17[3]: Mean values from 34 experiments evaluating apoptosis in erythroblasts

(includes 11 PV [V617F JAK2], 14 IE & 9 normals [all wild type JAK2, viable cell numbers for cytokine deprived, + Erythropoietin, + SCF normalised to Erythropoietin + SCF, standard error of the mean shown]



3.3 Discussion

When V617F JAK2 was described in PV there was wide variation from 65-97% in the prevalence of the mutation between studies. [Vainchenker et al, 2005] This variance may have been due in part to the sensitivity of the screening technique or perhaps due to the use of different diagnostic criteria (PVSG or WHO) in the individual studies. In some cases the explanation subsequently found was an alternative mutation in JAK2. [Scott et al, 2007] Yet another explanation is that some stored samples from apparently V617F negative JAK2 patients were, in fact, from patients who never actually had PV but who had been assigned this label in the absence of another clear cause for polycythaemia at the time.

The work described in this thesis provides data on a significant number (62) of patients with true polycythaemia including 27 with Polycythaemia Vera. This cohort of patients was closely assessed and investigated throughout the study period by myself and clinical data was regularly reviewed. As a result, several patients who had previously been diagnosed with PV (no other cause for erythrocytosis was apparent at the time) were subsequently 'undiagnosed', and 1 patient with previous borderline erythrocytosis was later diagnosed with PV. I am therefore confident that the scientific data reported in this study corresponds to the correct clinical diagnosis in these patients.

The PV patients in this cohort were broadly comparable with the general PV population having an average age of 61.5 years with similar clinical and laboratory profiles to that described in the literature: All PV patients in whom serum erythropoietin was tested had low/normal levels and all of the non-PV patients had levels in the normal or high range. High white cell count and platelet count was strongly associated with a diagnosis of PV. EECs were positive in 25/27 patients with PV. Of the 2 remaining PV patients, 1 did not have EECs performed and in the other, results from a single colony assay were equivocal. EECs were not positive in patients with IE/SP or normal controls.

A simple and robust method of screening for V617F JAK2 was used. The level of detection found with this method was consistent with this being a sensitive screening technique. Detection levels were comparable to the ARMS technique used in the study with the highest reported rate of V617F JAK2 positivity in PV (97%). [Baxter et al, 2005] Amongst the cohort of patients assessed in this study, 25/27 PV patients (93%) expressed V617F JAK2. One of the 2 negative results was from the patient with equivocal EECs who interestingly had a strong family history of probable myeloproliferative disease. Of the 25 patients expressing V617F JAK2, 20% showed homozygous expression which is comparable with the approximately 1/3 prevalence noted in published data.

The PRV-1 gene, a member of the uPAR receptor superfamily, was cloned and found to be associated with PV in 2000. [Temerinac et al, 2000] It was subsequently suggested that overexpression of this gene could completely separate PV from SP patients. [Klippel et al, 2003] Work done since this time has shown that PRV-1 can be upregulated in SP and that PRV-1 is not only homologous to NB1, (a gene overexpressed in reactive neutrophilia) but that both are alleles of the same gene now known as CD177. [Caruccio et al, 2006] PRV1 upregulation cannot therefore now be said to be absolutely specific for PV. Despite these more recent findings, it is still clear that PRV-1 upregulation defines a similar cohort of MPD patients to those with positive EECs and those expressing V617F JAK2. [Goerttler et al, 2005]

Screening for upregulation of PRV-1 using a semi-quantitative approach in this cohort showed that 24/27 patients with PV had marked (& 2/27 had mild) upregulation of PRV-1 expression. None of the 35 patients with IE had marked (though 6 had mild) upregulation of PRV-1. These PV patients therefore also show a high degree of correlation between identification of marked upregulation of PRV-1 with positive EECs and presence of the V617F JAK2 mutation.

In 2003, a group of patients with polycythaemia in Chuvashia, Russia were found to be homozygous for a 598 C>T mutation in the Von Hippel Lindau (VHL) gene resulting in an Arg200Trp substitution in the protein. [Ang et al, 2002] Autosomal dominant mutations of VHL have previously been described in association with cancers but this 598 C>T mutation was found to be associated with polycythaemia

and other clinical problems, but not with cancer. The pattern of inheritance for this mutation was recessive with only homozygotes being clinically affected. The VHL protein normally mediates proteosomal degradation of the α subunit of hypoxia inducible factor 1 (HIF-1) during cellular normoxia. This mutation disrupts binding of the VHL protein resulting in an accumulation of HIF-1α as normally occurs in cellular hypoxia. Chuvash polycythaemia is therefore a congenital disorder of oxygen sensing resulting in secondary polycythaemia and it is associated with elevated erythropoietin levels.

Following the original description, sporadic cases of Chuvash polycythaemia were reported around the world, [Percy et al, 2003; Bento et al, 2005; Perrotta et al, 2006] and elegant linkage analysis traced the mutation to a single founder effect which occurred from 1,000-62,000 years ago. [Liu et al, 2004] One of these reports included a Portuguese patient with heterozygous expression of the mutation who had polycythaemia in association with Ataxia-Telangiectasia. Other reports have identified alternative mutations in the VHL gene which may co-operate with the 598C>T mutation to cause polycythaemia. [Ang et al, 2002]

In the UCH cohort, 67 patient samples of neutrophil derived DNA, including 26 with PV, were screened for the Chuvash mutation using a restriction digest approach. One patient who screened positive was found to have heterozygous expression of the mutant which was confirmed by sequencing. No other mutations were detected in his VHL gene. This was an unexpected finding in a Caucasian British man of 52 years who had presented with apparent polycythaemia which was presumed to be secondary to the carcinoma of the larynx for which he was being treated. This patient was also a lifetime heavy smoker and serum erythropoietin was just above the normal range at 25.4 iu/l. The clinical relevance of the mutation in this case is unclear.

Work that has been published on the in vitro study of human erythroblasts includes cells which have been derived from mononuclear cells of the bone marrow, [Silva et al, 1998; Panzenbock et al, 1998; Myklebust et al, 2002; Ugo et al, 2004] from cord blood [Panzenbock et al, 1998], from cytokine mobilized progenitors [Somervaille et al, 2001] or from peripheral blood. [Wickenhauser et al, 2003; Dai et al, 2005, Arcasoy et al, 2005; Zeuner et al, 2006] In all studies, following manual separation,

mononuclear cells were cultured in a medium which preferentially enhanced erythroid growth. The culture medium components varied from study to study.

Some studies undertook selection of CD34+ stem cells 'up front'. [Ugo et al, 2004; Myklebust et al, 2002; Wickenhauser et al, 2003; Dai et al, 2005, Zeuner et al 2006; Somervaille et al, 2001, Zermati et al, 2001, Bouscary et al, 2003] Others relied on the differentiating medium principle originally described in a study by Sawada et al [1987] to generate erythroblasts within a population of mononuclear cells. Five of these studies used a delayed positive selection strategy, 3 initially used CD34+ selection followed by delayed CD36+ selection, [Ugo et al, 2004; Zermati et al, 2001, Bouscary et al, 2003] One study initially used CD34+ selection followed by delayed CD71+ selection, [Myklebust et al, 2002] and a further study used no initial selection but delayed Glycophorin A selection. [Silva et al, 1998] Four studies did not use additional delayed selection. [Wickenhauser et al, 2003; Somervaille et al, 2001; Arcasoy et al, 2005; Zeuner et al, 2006] One study, which built on previous work by members of this group, used a delayed negative selection strategy. [Dai et al, 2005]

Direct comparison of functional data between all the published studies described above may not be strictly appropriate since erythroblasts may functionally differ depending on whether they are derived from bone marrow or cord blood, cytokine mobilised cells or normal peripheral blood. [Van Epps et al, 1994] Direct comparison between studies using progenitors which have been derived from normal peripheral blood is probably more valid although different cell selection strategies may result in a subtly different cellular population. [Dai et al, 2005]

Initial experiments presented in this thesis explored delayed (to Day 6) positive selection of cells cultured in erythroid medium with either CD36 (thrombospondin receptor) or CD71 (transferrin receptor) conjugated microbeads. Neither of these markers was found to be specific: Most erythroblasts were CD36 and CD71 positive but some morphologically identical erythroblasts were not & contaminating cells were present in both the CD36 and CD71 positive fractions.

In subsequent experiments using cells derived from venesection packs, a technique involving delayed negative selection of primary mononuclear cells which had been

cultured in a medium preferentially enhancing erythroid growth was used. There were advantages and disadvantages with this approach: The clear advantage was that this 2-step procedure allowed harvest of all peripheral blood erythroblasts (including CD36 negative cells) with >95% purity at the end of the culture period. The major disadvantage was a laborious technique with several manual removal steps to remove unwanted cells.

In experiments using mobilised CD34+ progenitors from normal subjects cultured in erythroid medium, further selection was not performed. This approach resulted in a nucleated cell population which was only 80-90% erythroid which appears to be less than that found in the published data outlined above. It is possible that the lower purity in this study compared to that reported in published data related to the batch of fetal calf serum which was being used at the time.

The mixture of cytokines used for erythroid culture included Erythropoietin, Stem Cell Factor, Insulin Growth Factor 1, Interleukin 3, Estradiol and Dexamethasone. The roles of Epo, SCF and IL3 are illustrated in Figure 1[1] and their use for erythroid proliferation & controlled differentiation in vitro together with IGF1, Estradiol & Dexamethasone has been previously described [Panzenbock et al, 1998, Migliaccio et al, 2002]. Using this system, the Day 9 erythroblasts in this study were homogeneous morphologically and retained colony forming potential. Dexamethasone and estradiol were removed from the culture medium from Day 6 to allow a period of wash out for these pharmacological agents but high levels of erythropoietin, SCF, IL3 and IGF-1 were maintained. Of course, samples from all types of patients and normal controls in this study were treated identically, so that any artefactual changes induced by the in vitro culture system would apply to all diagnostic groups.

With this culture system, there was strong expression of CD71, intermediate expression of CD36, & Glycophorin A and weak expression of CD117 in Day 9 erythroblasts. These results are comparable with published data [Arcasoy et al, 2005; Josefsen et al, 2000] There was no significant difference in either immunophenotype (CD71, CD36 & Glycophorin A) or morphology of erythroblasts between subject groups, having looked at 43 Day 9 erythroblast specimens including 21 with PV.

The published literature on the differentiation status of PV erythroblasts relative to normal erythroblasts has not identified clear differences. Whilst one group found marginally increased expression of Glycophorin A in PV, [Ugo et al, 2004] other groups [Dai et al, 2005; Zeuner et al, 2006] have found no differences. Corroboration of the immunophenotype findings in this study using molecular gene expression profiling of our samples was obtained & these results, discussed in Chapter 6, confirm the FACS findings of no significant difference in erythroblast differentiation between PV & controls.

The colony forming potential of cultured erythroblasts was assessed by taking cells on each day of culture and plating them into standard colony assays. There was predominantly BFUe formation up to Day 4 and subsequently predominantly CFUe formation up to and including Day 10 of liquid culture. There was marked expansion of erythropoiesis by Days 4-6 which is illustrated in Figure 12[3] and corresponds to published findings. [Panzenbock et al, 1998]

Polycythaemia Vera is associated with an increased white cell and mononuclear cell count. When compared with patients with myelofibrosis, the number of circulating stem cells derived from the mononuclear cell compartment is not significantly increased. [Passamonti et al, 2003] However, when patients with PV are compared with normal controls, the number of circulating CD34+ cells is increased. [Andreasson et al, 1997] Within the group of PV subjects who express V617F JAK2 there is a dose-response effect between the number of mutant alleles and the concentration of circulating CD34+ progenitors. [Passamonti et al, 2006] CD34+ stem cell progenitors derived from patients with PV are reduced in patients taking therapeutic Hydroxycarbamide. [Andreasson et al, 2000]

In this cohort of patients, an elevated white cell count was strongly associated with a diagnosis of PV. Presumably as a result of this, there was an increased yield of mononuclear cells from PV patients compared to other groups with the notable exception of patients taking therapeutic Hydroxycarbamide. In addition, there was a trend towards increased yield of mononuclear cells in PV patients with homozygous rather than heterozygous expression of V617F JAK2.

Publications that have looked at proliferation in erythroblasts have found increased proliferation with a spectrum of added cytokines in PV. [Ugo et al, 2004; Wickenhauser et al, 2003; Dai et al, 2005; Zeuner et al, 2006] Comparison of erythroblast yield with the number of mononuclear cells initially harvested from peripheral blood in this study showed that the proportion of erythroblasts produced from PV patients with homozygous expression of V617F JAK2 was 3.5 times higher than normal controls.

Jamieson et al [2006] compared erythroid colony formation in CD34+ cells derived from patients with PV and normals including bone marrow, cord blood and peripheral blood specimens. They found a significant difference in the quantity and quality of erythroid but not myeloid colonies. Whilst it had been recognized that the ratio of erythroid to myeloid progenitors in the bone marrow was increased in PV, [Anger et al, 1989] earlier studies had not shown a significant increase in erythroid (compared to myeloid) committed stem cells as assessed by colony assays. [Eaves et al, 1980]

In this study, 80 colony assays were performed using peripheral blood mononuclear cells including 33 from patients with PV, 37 from patients with IE and 10 normal controls. Erythroid colonies were increased in PV relative to normal individuals, but not relative to IE; erythroid:myeloid colony ratios were not altered in PV compared to either IE or normals. These results do not suggest skewing of CD34+ cells to erythroid commitment in PV.

It has been suggested that apoptosis is reduced in PV erythroblasts. This observation principally stems from a study by Silva et al which reported increased expression of the anti-apoptotic protein, Bcl-xL in PV erythroblasts compared to controls. This study used erythroid progenitors derived from PV bone marrow with growth in erythroid medium and the authors also reported reduced morphological evidence of apoptosis with erythropoietin deprivation in representative PV samples [Silva et al, 1998]. Bcl-xL may not, however, play a role that is limited to apoptosis (BCL-XL is progressively upregulated during erythropoietin and SCF induced erythroblast differentiation in vitro). [Myklebust et al, 2002] Garcon et al [2006] have described induction of EECs from erythroid progenitors with overexpression of STAT/Bcl-xL which is reversed by knock down of STAT5 & Bcl-xL suggesting that Bcl-xL may

play a role in proliferation. Sui et al [2000] specifically looked at differences in erythropoietin and SCF induced expression of Bcl-xL in erythroblasts and the relationship of this to apoptosis with Wortmannin (PI3K inhibitor) and found that differential Bcl-xL expression did not correlate with apoptosis but with proliferation and differentiation. Josefsen et al [2000] found that Bcl-xL expression increased with days of in vitro culture but that this did not correlate with a change in apoptosis.

Two published studies have taken day 7/8 bone marrow derived erythroblasts from liquid culture and shown that, in some cases, EECs can be derived from this cellular population. [Silva et al, 1998, Ugo et al, 2004]. In order to examine whether PV erythroblasts cultured in vitro retain their relative erythropoietin independence, the work presented in this chapter looked at samples incubated overnight with and without erythropoietin. MTS assays to quantify viable cell number showed that relative resistance to erythropoietin deprivation was a feature of V617F JAK2 mutated erythroblasts. In addition, homozygous expression of the mutant form was associated with increased resistance to erythropoietin deprivation compared to heterozygous counterparts.

Measurement of apoptosis in erythroblasts deprived of erythropoietin or SCF using Annexin V-FITC staining of erythroblasts and FACS analysis showed no difference between PV and control cells. These findings are similar to Panzenbock et al [1998]. Other groups have also found no difference in functional erythroblast apoptosis in PV: Ugo et al [2004] found no difference between PV & controls in the apoptosis of erythroblasts with erythropoietin deprivation using Annexin V methodology. Dai et al [2005] reached similar conclusions using TUNEL assays.

Polycythaemia Vera is a disease characterised by erythroid expansion. There are 3 principal potential explanations for this phenomenon (which are not mutually exclusive): The proportion of circulating stem cells committed to erythroid differentiation could be higher, erythroblast proliferation could be increased or erythroblast apoptosis could be reduced. In order to better understand the pathophysiology of PV, an in vitro model was developed which allowed direct comparison of the function of erythroid progenitors derived from patients with PV, Idiopathic Erythrocytosis (disease entities characterised by erythroid expansion not

due to PV) and normal controls. Results obtained from this did not suggest erythroid skewing of the stem cell compartment in PV. There was significantly increased proliferation of PV erythroblasts in the presence of maximal concentrations of cytokines as well as increased viable cell number, but no alteration of apoptosis, in PV erythroblasts which had been cytokine-deprived.

CHAPTER 4 – RESULTS 2

Signal transduction pathways in primary erythroid progenitors & haematopoietic tumour cell lines

4.1 Signal transduction pathways activated by SCF and Epo differ between haematopoietic tumour cell lines & primary human erythroid progenitors

Work shown in this chapter includes study of the signalling enzymes AKT, p70S6K, GSK3a/b, p38MAPK, RSK, ERK, JAK2, STAT5 & Pim-1. Activation of phosphoinositide 3-kinase signalling leads to subsequent phosphorylation and activation of the Akt and p70S6 Kinases. Active Akt can phosphorylate and inactivate GSK3alpha/beta. However, GSK3 can be a downstream target of the PI3K, MAPK or Protein Kinase C (PKC) pathways [Doble & Woodgett, 2003]. Activation of Ras signalling by growth factors leads to the subsequent activation of the ERK MAPkinase – a downstream target for ERK is the p90RSK. Janus-associated kinases (JAKs) directly activate Signal Transducers and Activators of Transcription (STATs) of which Pim-1 oncogene (Pim-1) is a downstream target [Stout et al, 2004].

Previous work by our group identified PI3K mediated phosphorylation of GSK3 as being critical for erythroblast survival [Somervaille et al, 2001]. The work presented in this chapter initially explored the precise position of GSK3 phosphorylation via PI3K and other pathways in haematopoietic cell lines and then moved on to work with primary erythroid precursors derived from the peripheral blood of patients with Polycythaemia Vera and control subjects. To enable study of the contribution of individual pathways, small molecule inhibitors were used and a representation of their effects together with the position of GSK3 in the pathways studied is shown in Figure 1[4] & Table 1[4].

Figure 1[4]: Simplified representation of the signalling pathways and small molecule inhibitors used in this chapter

(PKC = Protein Kinase C, MAPK = mitogen-associated protein kinase, PI3K = phosphoinositide-3-kinase, JAK = Janus-associated kinase, STAT = Signal transducer and activator of transcription, Pim = Pim oncogene, GSK3 = Glycogen synthase kinase 3, inhibitors target the indicated pathways with Go6976 inhibiting both JAK & PKC and LY294002 inhibiting PI3K a & Pim)

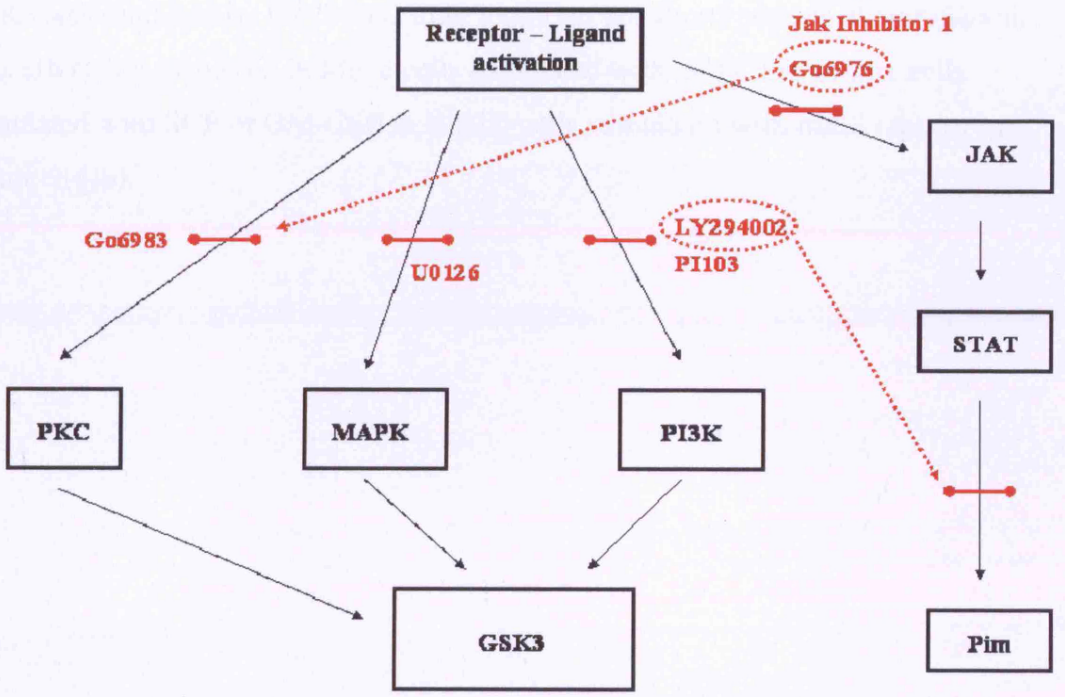


Table 1[4]: Small molecule inhibitors of signalling pathways

(LY294002* also inhibits Pim-1 kinase activity)

Pathway	PKC	MAPK	PI3K	JAK
Inhibitor 1	Go6983	U0126	LY294002*	Jak Inhibitor 1
Inhibitor 2	Go6976		PI103	Go6976

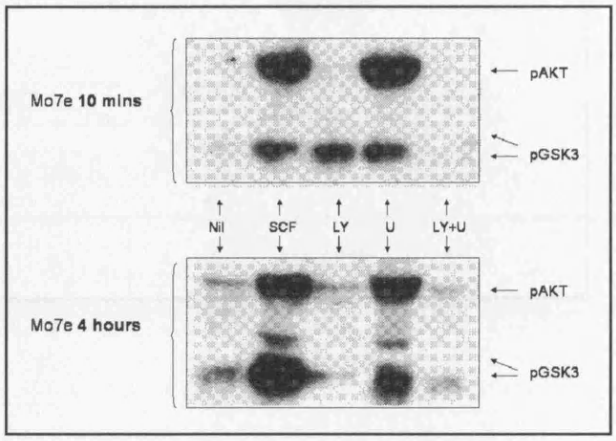
4.1.1 The kinetics of individual pathway stimulation & inhibition differ

The phosphorylation of GSK3 via PI3K and MAPK was initially explored in haematopoietic cell lines: Figure 2[4]a shows the effects of stimulation of Mo7e cells with SCF together with inhibition of either or both pathways. As expected, pAKT was inhibited by LY294002 alone but not by U0126 alone for both short and long periods of stimulation (note the combination of LY294002 & U0126 showed greater inhibition than that seen with LY294002 alone). By contrast, phosphorylation of GSK3 was inhibited by LY294002 after long (but not short) periods of stimulation. This effect was also seen in Mo7e cells stimulated with GM-CSF, in TF1 cells stimulated with SCF or GM-CSF & in 32D cells stimulated with mIL3 (shown in Figure 2[4]b).

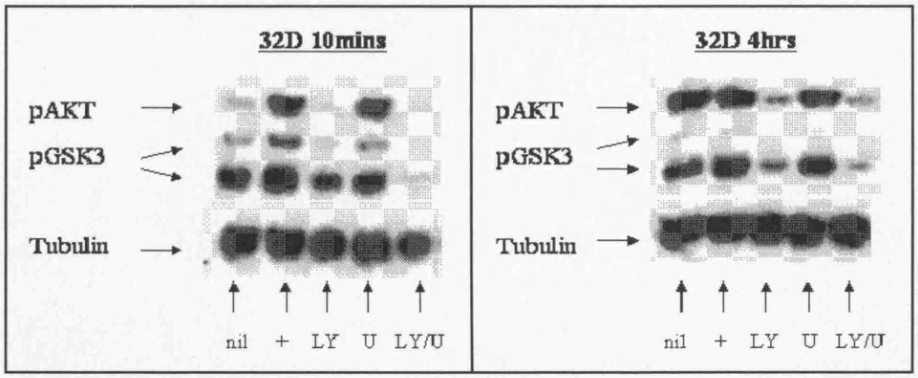
Figure 2[4]: The early phase of GSK3 phosphorylation is dependent on both PI3K & MAPK pathways in haematopoietic cell lines whereas the late phase is PI3K dependent only

(**a.** Mo7e cells were starved of cytokines overnight then incubated with or without LY294002 and/or U0126 followed by stimulation with SCF for 10 mins or 4 hrs, western blots from protein lysates probed for pAKT, pGSK3 & tubulin (not shown) **b.** 32D cells starved then stimulated with IL3 +/-LY294002 or U0126 for 10mins & 4 hrs, western blots from protein lysates probed for pAKT, pGSK3 & tubulin)

a.



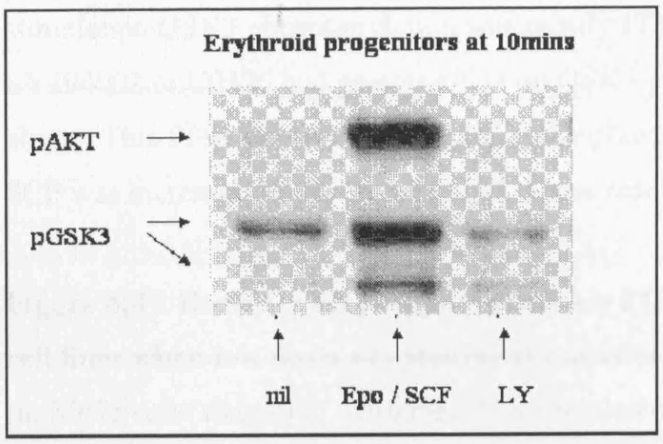
b.



In contrast, Western blots from erythroid progenitors showed (as our group previously reported), that phosphorylation of GSK3 was entirely PI3K dependent at early time points (10 mins of cytokine stimulation as shown in Figure 3[4]).

Figure 3[4]: Phosphorylation of GSK3 is immediately PI3K dependent in erythroid progenitors

(erythroblasts derived from peripheral blood after 9 days of in vitro culture washed and starved in serum free medium for 2 hours then stimulated with either Epo or SCF [SCF shown] for 10 mins +/- pre-incubation with LY294002)



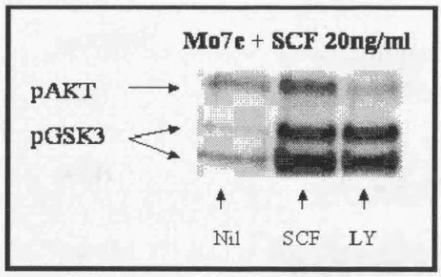
4.1.2 Cytokine dose affects pathway activation in cell lines

A potential explanation for the partial PI3K dependency of GSK3 phosphorylation observed in haematopoietic cell lines relates to the supraphysiological doses of cytokines used in previous experiments. Subsequently Mo7e cells were stimulated with a range of cytokine concentrations. When low doses of SCF were used for stimulation GSK3 phosphorylation was mostly PI3K dependent (the combination of LY294002 & U0126 had greater effect on GSK3 phosphorylation than LY294002 alone). This PI3K dependency of GSK3 phosphorylation was lost when the dose of SCF was increased to 5ng/ml or above. These results are presented in Figure 4[4].

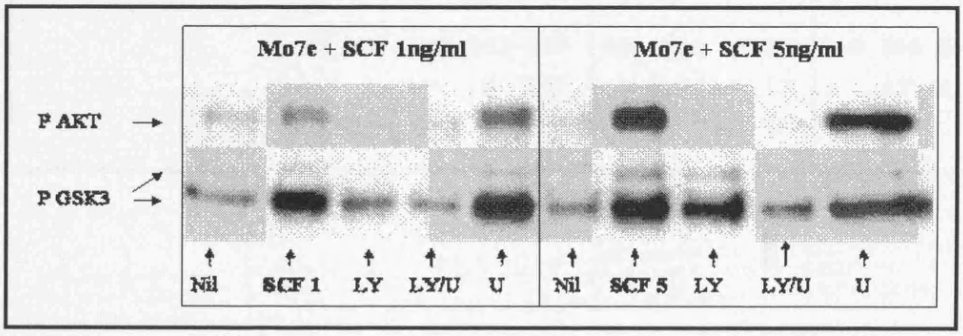
Figure 4[4]: Early GSK3 phosphorylation is PI3K dependent in haematopoietic cell lines when low doses of cytokine stimulation are used

(**a.** Mo7e cells starved of cytokines then stimulated for 10 mins with SCF 20ng/ml +/- LY294002 [LY], western blots from protein lysates probed for pAKT and pGSK3, **b.** Mo7e cells starved of cytokines then stimulated for 10 mins with SCF 1ng/ml or 5ng/ml +/- LY294002, U0126 [U] or both, western blots from protein lysates probed for pAKT and pGSK3)

a.



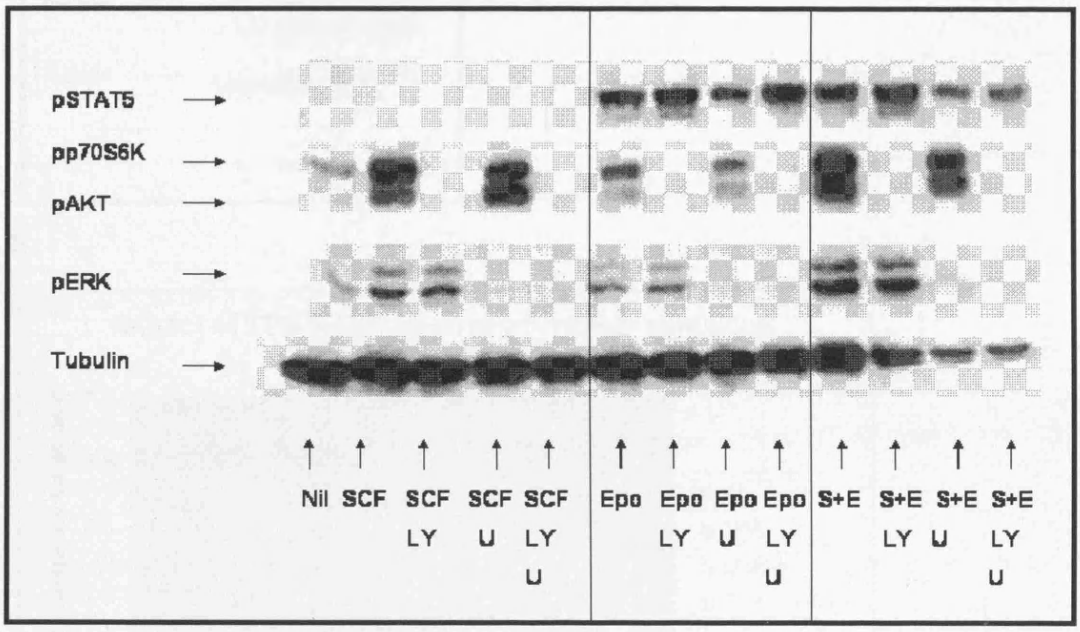
b.



4.1.3 Cytokines may show overlapping pathway activation patterns in erythroid progenitors

A possible explanation for the observation that high doses of cytokines induced pGSK3 via PI3K and MAPK in haematopoietic cell lines, but not in primary erythroid cells, was that Epo did not induce MAPK phosphorylation in erythroid cells. However, as shown in Figure 5[4], SCF or erythropoietin stimulation of erythroid progenitors did result in activation of MAPK. This figure also shows that phosphorylation of STAT5 was induced by erythropoietin (but not SCF) in erythroid progenitors.

Figure 5[4]: MAPK is phosphorylated by both Epo & SCF whilst STAT5 is independently phosphorylated by Epo but not SCF in erythroid progenitors (western blots from primary erythroblasts starved then incubated with no inhibitor, LY294002 or U0126 or both, then [with the exception of the first lane] stimulated with SCF, erythropoietin or both cytokines for 10 mins, blots probed for the known PI3K targets pAKT & pp70S6K, pERK, pSTAT5 & tubulin)

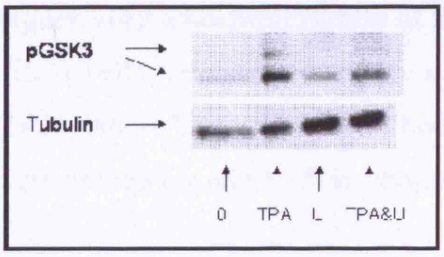


In order to further test the activity of the MAPK pathway in erythroid progenitors, cells were stimulated with tetradecanoyl phorbol-myristate acetate (TPA) which can potently activate MAPK signalling via direct activation of protein kinase C. Under these conditions, phosphorylation of GSK3 was predominantly MAPK dependent as shown in Figure 6[4] a&b. It is therefore apparent that the MAPK pathway can be stimulated to induce pGSK3 in erythroid progenitors but that this mechanism is not usually engaged by SCF or Epo.

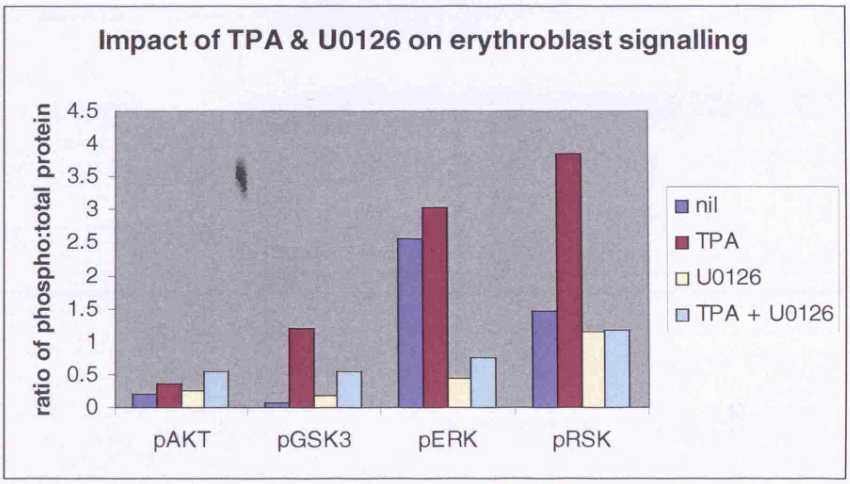
Figure 6[4]: TPA can induce phosphorylation of GSK3 in primary erythroblasts via the MAPK pathway

(western blots of protein lysates from erythroid progenitors which had been starved, stimulated with tetradecanoyl phorbol-myristate acetate [TPA] for 10 mins, incubated with the MAPK inhibitor U0126 or treated with both TPA and U0126. Blots for pGSK3 and Tubulin are shown in **a),** scanning densitometry results relative to tubulin from blots for pAKT, pGSK3, pERK & pRSK [also MAPK pathway] are shown in **b**)

a.



b.

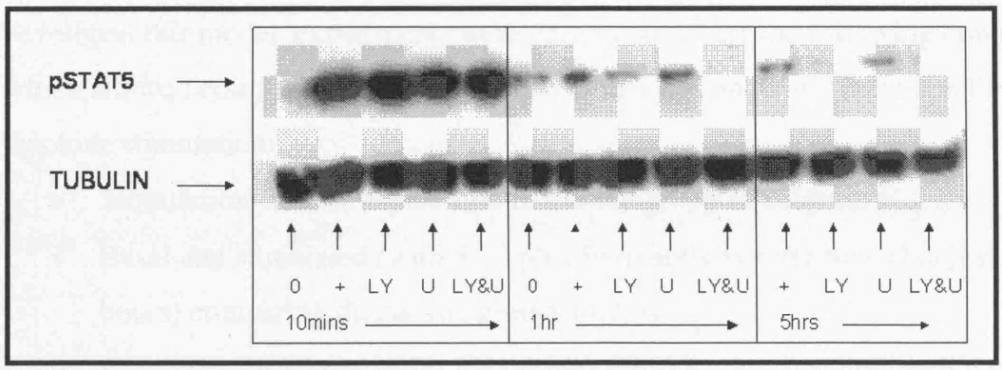


4.1.4 There may be cross-talk between signalling pathways revealed by inhibitor studies

In several experiments using Mo7e cells stimulated with SCF, GM-CSF, Epo & Thrombopoietin , there was apparent cross-talk between the PI3K & MAPK pathways: Whilst LY294002 blocked pAKT & U0126 blocked pERK as expected, there was a paradoxical increase in pAKT when cytokine stimulated cells were pre-incubated with U0126 alone (data not shown). Limited experiments in erythroid progenitors were inconclusive on this issue (see representative study in Figure 5[4]).

In Mo7e cells it was also observed that although tyrosine phosphorylation of STAT5 was overwhelmingly PI3K & MAPK independent at early time points, with longer periods of stimulation, a weak pSTAT5 signal showed evidence of cross talk with the PI3K pathway as shown in Figure 7[4]. There was insufficient time to pursue this issue specifically in erythroid progenitors.

Figure 7[4]: Phosphorylation of STAT5 can be PI3K dependent
(Mo7e cells were starved and stimulated with GM-CSF +/- pre-incubation with LY294002 [LY], U0126 [U] or both for 10 minutes, 1 hour or 5 hours, western blots were probed for pSTAT5 & tubulin)



4.2 Basal & stimulated primary erythroid progenitor signalling

As discussed previously, evidence published in the scientific literature pointed towards abnormalities in signal transduction in Polycythaemia Vera as being key to the pathophysiology of the disease. This work therefore set out to define these abnormalities in individual pathways using erythroid progenitors derived from patients with PV comparing them with control subjects. Primary erythroid progenitors were derived from peripheral blood venesection packs and from normal buffy coat residues supplied by the National Blood Service as described in 2.2.1-5. After a period of in vitro culture with a negative selection step, mononuclear cells were assessed morphologically and by immunophenotype and samples with >90% erythroblasts were processed further.

Basal (unstimulated) activity and cytokine stimulated activitation of signalling pathways was investigated with an emphasis on comparisons over time as this issue had been relevant in our previous work. Western blot exposure times were selected for non-saturating signals allowing comparison between unstimulated & stimulated lanes referenced against the total loading protein control.

In order to study signalling, a reproducible model of erythroid development was established which would allow ready comparison between groups (4.2.1). Having developed this model, experiments were carried out under the following conditions which allowed comparison between diagnostic groups under different conditions of cytokine stimulation:

- Stimulation with SCF plus Epo in a small group of subjects (4.2.2).
- Basal and stimulated (with SCF plus Epo) activity over time (but less than 6 hours) comparing diagnostic groups (4.2.3).
- Basal and stimulated (with Epo alone) activity over time comparing diagnostic groups (4.2.4)

Using data from the the total set of experiments a comparison in basal signalling between PV and control groups was made (4.2.5). Finally, differences between diagnostic groups with prolonged (>6 hour) stimulation times were investigated (4.2.6).

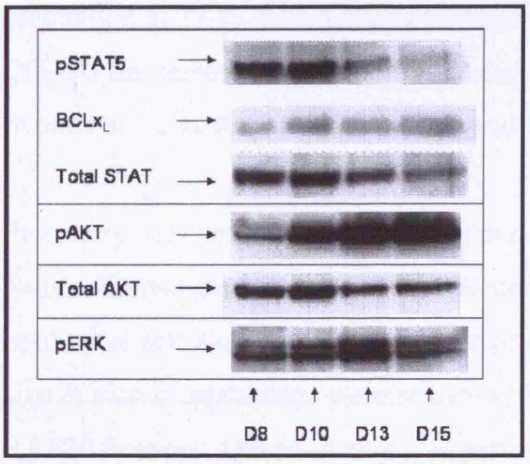
4.2.1 Establishing a basis for investigating erythroblast signalling

In a first series of experiments, the effect of the number of days of in vitro culture (in standard growth factor replete conditions) on signal transduction in the PI3K, MAPK & STAT pathways was examined. BCLxL expression, as being both a potential STAT5 target and an erythroid differentiation marker, was measured in parallel. A representative study using erythroblasts from a patient with IE is shown in Figure 8[4].

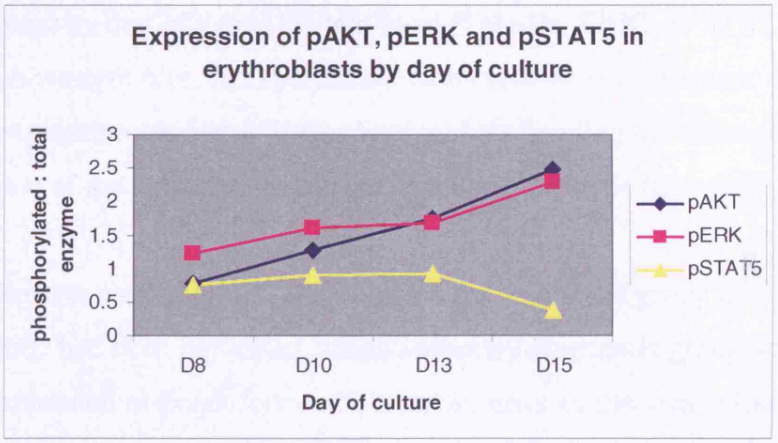
Figure 8[4]: Assessing activity of the PI3K, MAPK & STAT pathways in erythroid progenitors by day of in vitro culture

($2x10^6$ cells taken direct from culture on days 8, 10, 13 & 15, protein lysates from western blots incubated with antibodies to pAKT, pERK & pSTAT5 & BCLxL followed by Total STAT & AKT [**a**], densitometric assessment of phospho-enzymes relative to total protein shown [**b**])

a.



b.



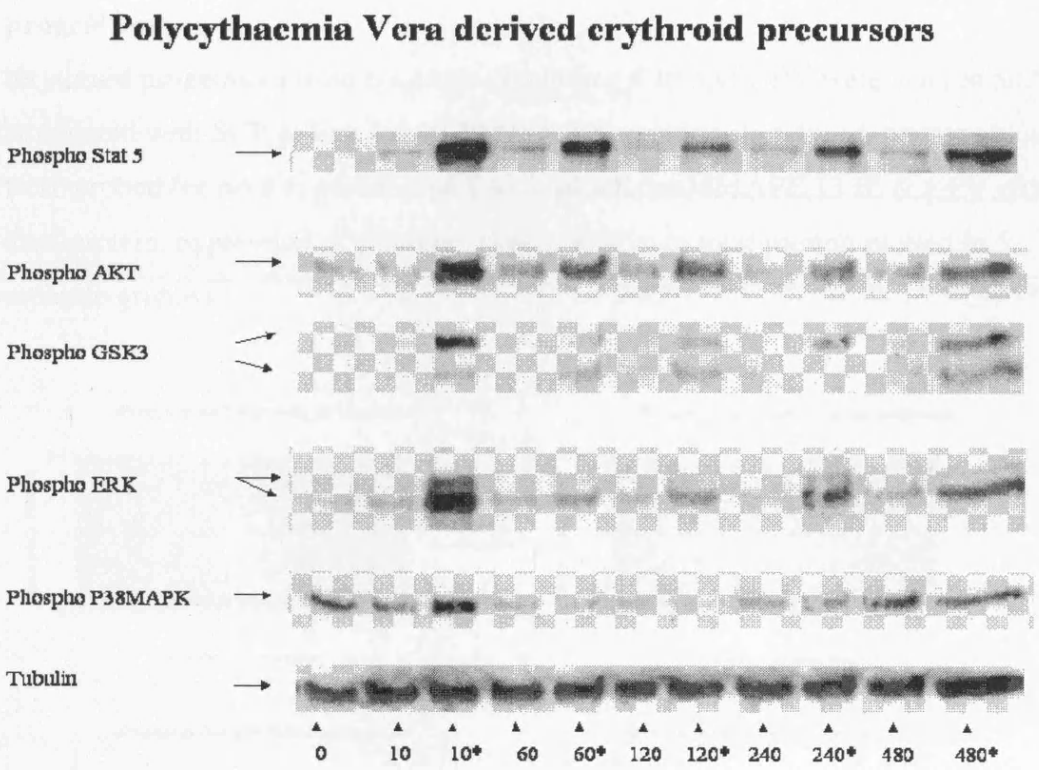
These experiments confirmed that, in order to make accurate comparison between erythroblasts by diagnostic group, it would be optimal to select a specific day of culture and stick with it throughout experiments. Day 9 was chosen for all future work as this would allow assessment of each of the 3 signalling pathways that were the focus of this work to be studied and because this day coincided with an optimal number of erythroblasts available.

Subsequent methodology is described in 2.6.1-2 but in brief: erythroblasts were washed free of cytokines and suspended in serum free medium for 2-3 hours at 37^0C & 5% CO_2. Cells were then aliquoted into the desired number of experimental samples with 0.5-1 x 10^6 cells per point each suspended in 1ml medium. Where appropriate, inhibitors were then added followed by further incubation at 37^0C for 30-60 minutes. Cytokines were then added to selected samples at concentrations of 1iu/ml for Epo and 10ng/ml for SCF (previous experiments showed that phosphorylation was saturated with these doses of cytokines) followed by further incubation at 37^0C. If this incubation step was >1 hour, cells were maintained at 5% CO_2. At the selected time point following cytokine stimulation, (10 minutes to overnight) cells were rapidly chilled and protein lysates made.

Protein lysates were then assessed as per 2.6.4 – 2.6.7 by standard western blotting. Nitrocellulose membranes were incubated sequentially with a range of Phospho-antibodies followed by an appropriate protein loading control. Bands of the correct size & visual appearance were scanned from Hyperfilm with an Epson Stylus Photo RX620 Scanner. Densitometry was performed using NIH ImageJ software. A ratio of protein expression was derived by dividing the densitometric value for the phospho-band by that of a total protein band (Tubulin, ERK2 or STAT5b) for the selected lane on western blot. In experiments with cytokine stimulation $\geq$ 4 hours, this protein expression ratio was further analysed by dividing the value for the basal sample by that of the cytokine stimulated sample at the same time point.

Results were assessed according to the diagnostic group of the patient from whom they had been harvested. Mean values by diagnostic group were calculated and are presented in graph form with standard error of the mean plotted. Representative western blot results from one patient are shown in Figure 9[4].

Figure 9[4]: Illustrative western blot results from erythroblasts derived from a patient with Polycythaemia Vera starved and stimulated over a time course
(Day 9 erythroblasts were washed and starved for 2 hours in serum free medium at $1x10^6$ per ml, half of the 1 ml aliquots were stimulated with Epo plus SCF, starved & stimulated [*] aliquots were lysed in parallel after 10, 60, 120, 240 & 480 minutes, western blots were made and the membrane incubated sequentially with phospho-antibodies prior to assessment of total protein)

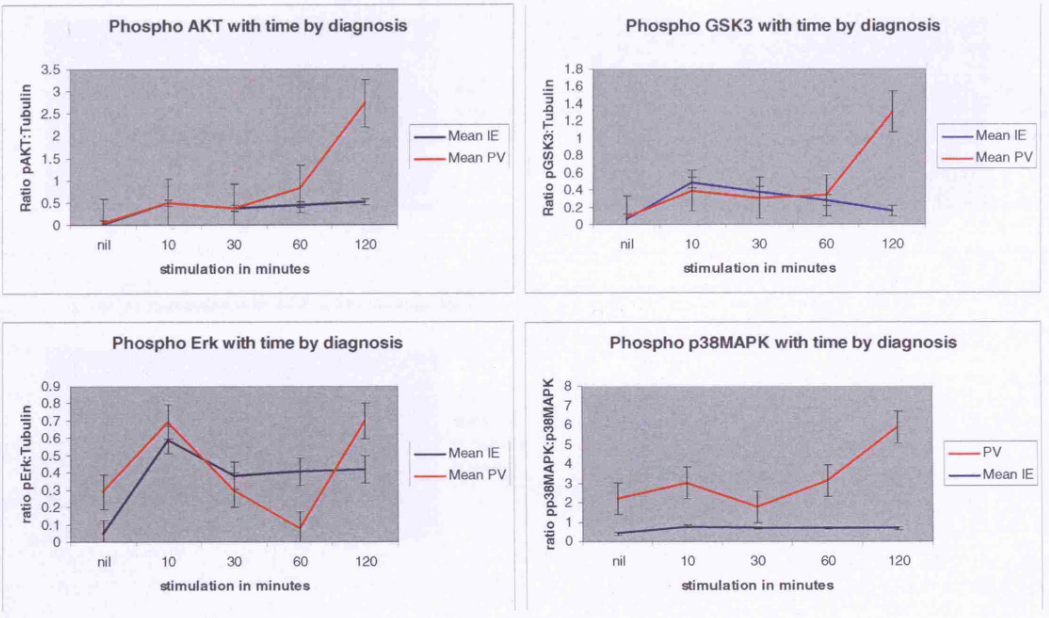


4.2.2 Protein phosphorylation patterns in erythroblasts stimulated over a short time course

Initial experiments compared activity in 6 samples of erythroid progenitors including 2 from patients with PV. Analysis of the PI3K, STAT & MAPK pathways is shown in Figure 10[4]. Results for pp38MAPK are derived from only 4 experiments (3 IE, 1 PV) but are shown to provide comparison with pERK.

Figure 10[4]: Quantification of signal pathway activation in stimulated erythroid progenitors

(erythroid progenitors from 6 samples including 4 IE and 2 PV were starved and stimulated with SCF & Epo for 10,30 60 & 120 minutes then lysed, western blots were probed for pAKT, pGSK3, pSTAT5, pERK, pp38MAPK [3 IE & 1 PV only] & total protein, expression of phosphorylated relative to total protein plotted in 5 separate graphs)



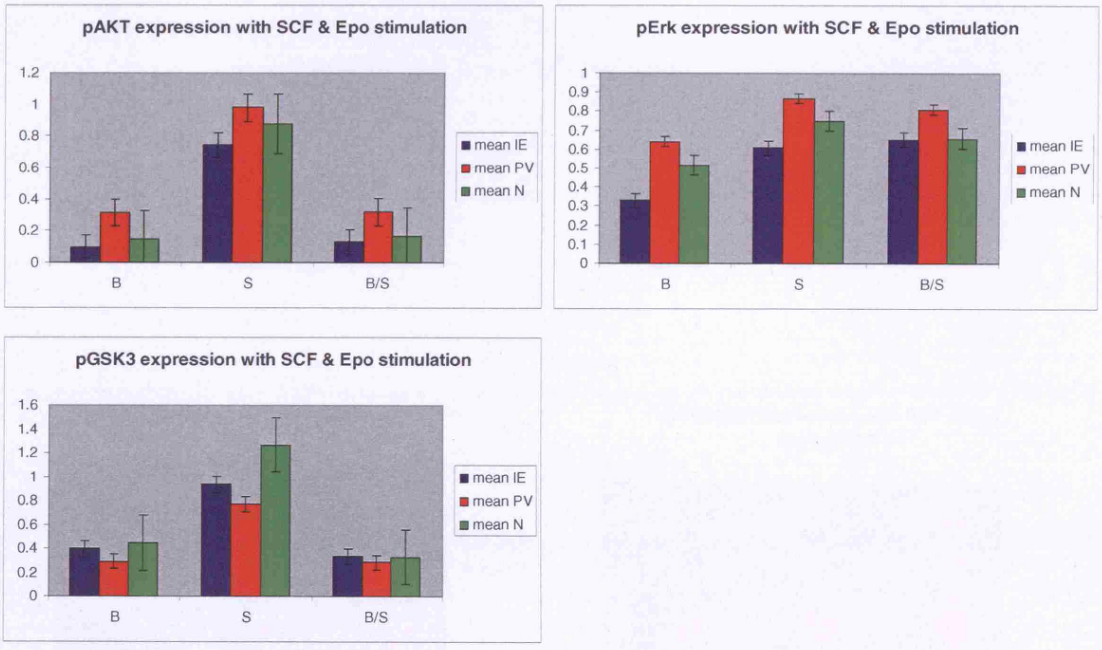
These preliminary results from a small group of patients established a working platform and suggested that there may be some differences between PV and IE in both the basal and stimulated signalling pathways of erythroblasts.

4.2.3 Protein phosphorylation patterns in erythroblasts both starved and stimulated for a short time (<1hour) with erythropoietin and SCF

In order to separate out the alterations in signalling between basal and short-term cytokine stimulated responses a larger cohort of 17 experiments using erythroid progenitors from patients with PV & controls was carried out. Average results according to diagnostic group are shown in Figure 11[4] & 12[4].

Figure 11[4]: Comparing basal & stimulated pAKT, pERK & pGSK3 between PV & controls

(mean values from 8 patients with IE, 7 patients with PV & 2 normals shown, protein band expressed relative to total protein loading control for basal [B] & stimulated [S = SCF+Epo] samples with the ratio of basal/stimulated [B/S] in the 3rd column, standard error of the mean plotted)

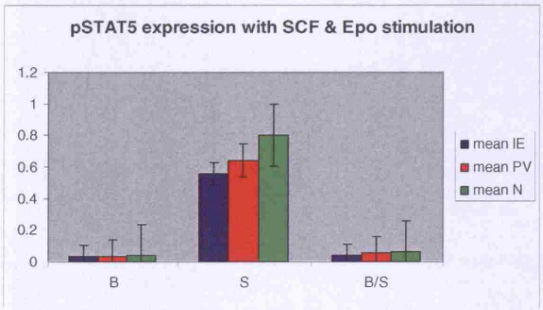


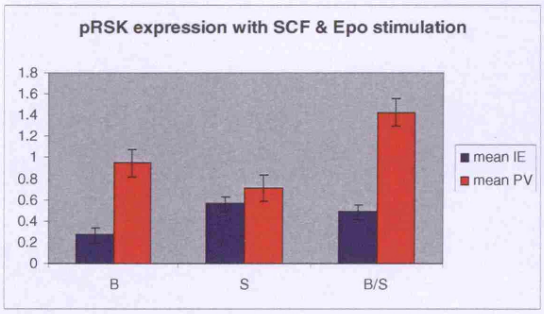
This pattern suggested that there was increased basal and stimulated phosphorylation of AKT & ERK in the PV subjects compared to IE. Results from the normal samples were probably similar to the IE patients but could not be accurately placed due to the low number of samples available. Interestingly GSK3 phosphorylation was not simply aligning with AKT phosphorylation.

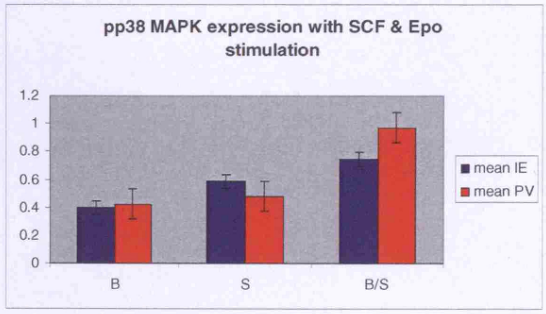
For some of these patients results were available for pSTAT5, pRSK and pp38 MAPK and these are shown in Figure 12[4]. Whilst results for RSK and p38MAPK could fit with the ERK phosphorylation pattern, the pattern for STAT5 was different in this smaller group: basal phosphorylation was not significantly increased and STAT5 was not excessively stimulated in the PV subjects.

Figure 12[4]: Comparing basal & stimulated pSTAT5, pRSK and pp38 MAPK between PV & controls

(mean values from diagnostic groups shown, protein band expressed relative to total protein for basal [B] & stimulated [S = SCF+Epo] samples with the ratio of basal/stimulated [B/S] in the 3rd column, standard error of the mean plotted, includes the same group of experiments as for Figure 11[4] but results available for fewer patients – pSTAT5 = 13 [7 IE, 4 PV, 2 normals], pRSK = 5 [2 IE, 3 PV] & pp38MAPK = 8 [5 IE, 3 PV])





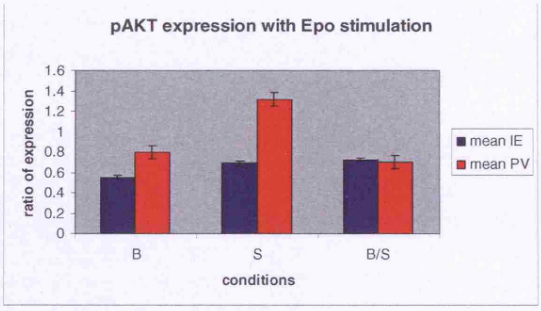


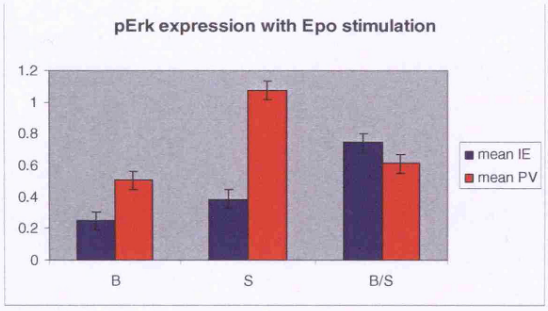
4.2.4 Protein phosphorylation patterns in erythroblasts both starved and stimulated for a short time (<1hour) with erythropoietin alone

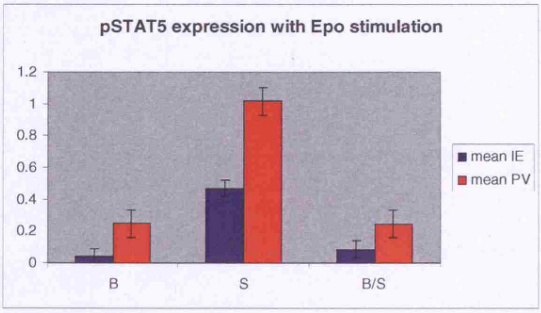
In order to assess whether the differences by diagnostic group were due to Epo-dependent abnormalities, as Epo signals via Jak and SCF characteristically does not, further experiments from 16 subjects including 9 with PV in which cytokine stimulation was with Epo alone were done. This increased number of samples could potentially have drawn out small differences in signalling by assessing a larger cohort of patients with PV. Average results by diagnostic group are shown in Figure 13[4]. There was no difference between the PV samples heterozygous & homozygous for V617F JAK2.

Figure 13[4]: Relative phosphorylation in erythroblasts starved or stimulated with erythropoietin for <1hour

(Averaged results from 16 experiments shown in 3 separate graphs of pAKT, pSTAT5 and pERK, includes 7 samples from patients with IE [all wild type for JAK2] and 9 samples from patients with PV [2 homozygous, 7 heterozygous for V617F JAK2], expression of phospho-antibodies relative to total protein for basal [B] & stimulated [S] samples shown with basal/stimulated ratio [B/S], standard error of the mean plotted)







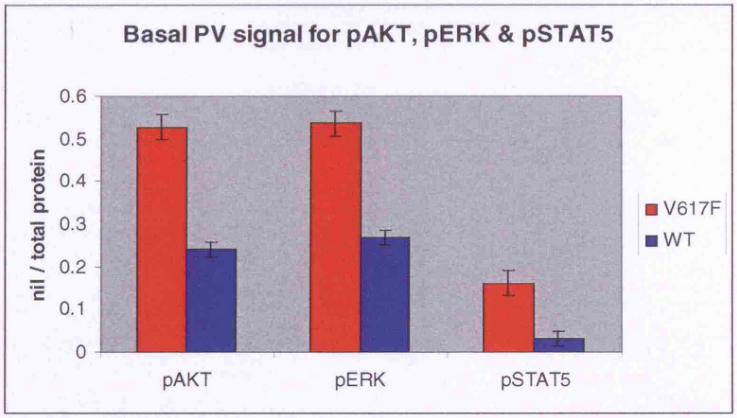
These findings confirmed that basal and stimulated (for <1hour) expression of pAKT and pERK were higher in PV. The larger cohort of samples also brought out the slight increase in basal and stimulated pSTAT5 in PV. Interestingly the ratios of expression between basal and stimulated samples were somewhat different when Epo was used alone for stimulation: The ratios for pAKT and pERK were normal and only the ratio for pSTAT5 was increased. This may indicate that signalling abnormalities in PV are not entirely JAK2 dependent.

Since the basal signal in all the preceding experiments was derived from cells under the same conditions (erythroblasts washed and starved in serum free medium for 2-3 hours) all this data was merged showing that basal pAKT, pERK and pSTAT5 were significantly higher in PV than in control subjects but that there was variation between individuals as shown in Figure 14[4].

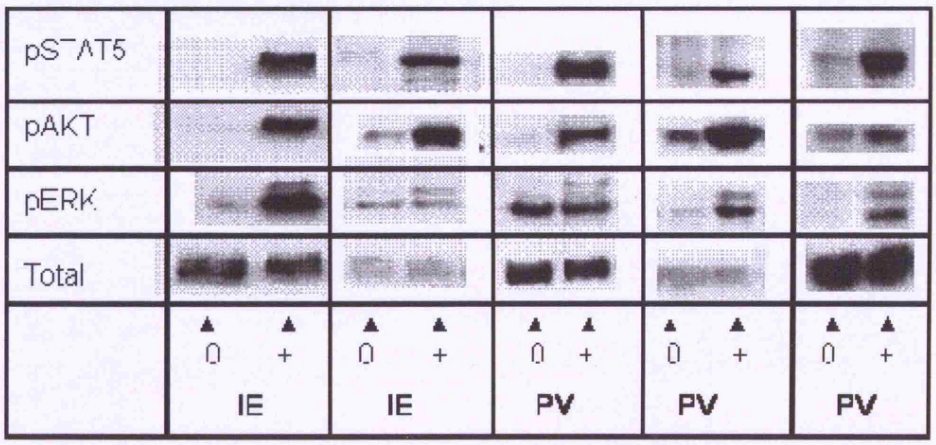
Figure 14[4]: Basal signalling of PV erythroid progenitors is increased in all pathways

(**a.** western blots from 18 patients with PV [V617F JAK2] & 21 controls [wild type WT JAK2] showing the erythroblast signal after 2-3 hours of starvation in serum free medium for pAKT, pERK & pSTAT5 relative to total protein, standard error of the mean shown, **b.** representative blots from 2 patients with IE and 3 with PV showing starved & stimulated phospho-enzymes relative to total protein. **c.** all values for basal signal relative to total protein shown with mean of all portrayed [pSTAT5 values multiplied by 5 & maximum value adjusted from 6.4 to 3.1 for representation])

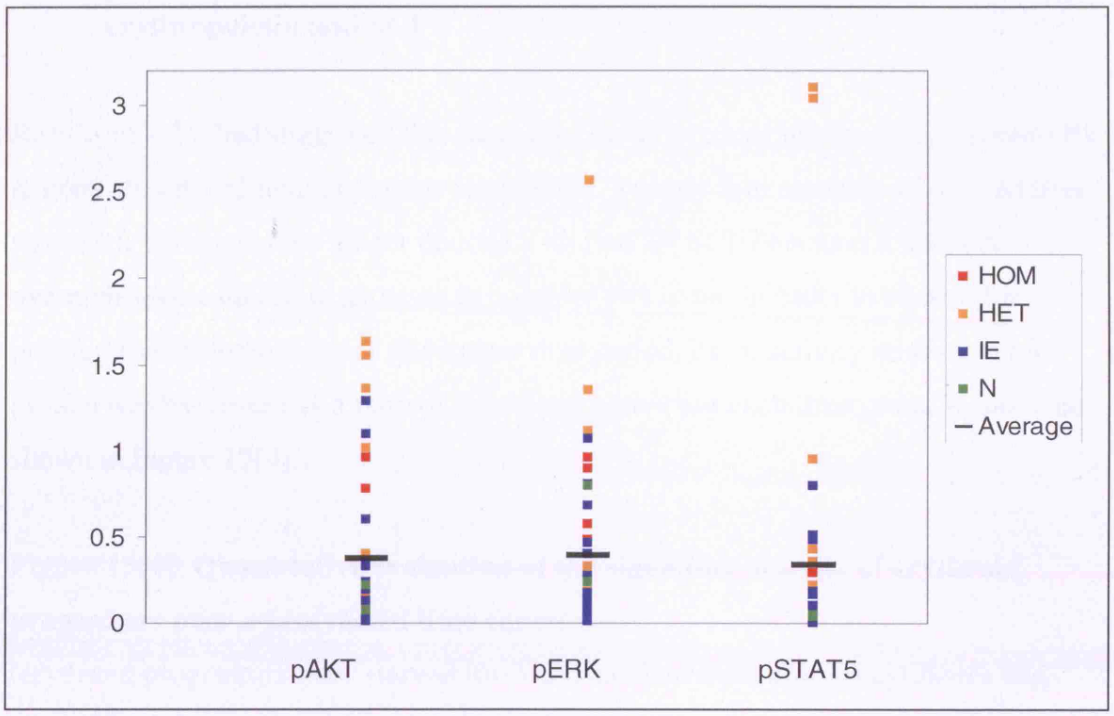
a.



b.



c.

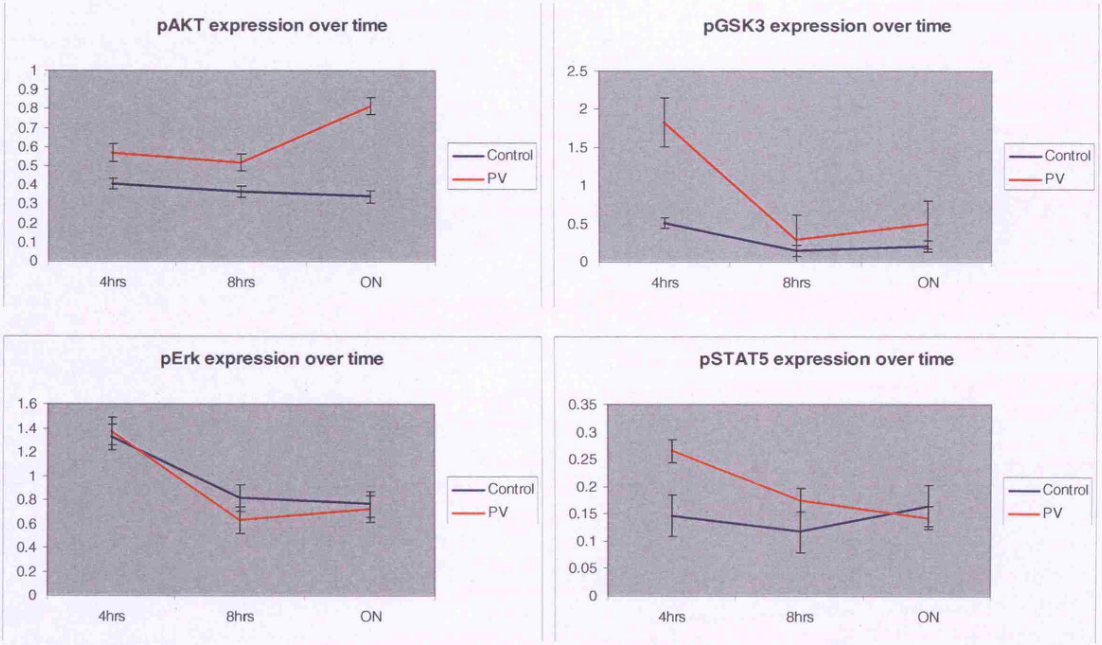


4.2.5 Protein phosphorylation patterns in erythroblasts both starved and stimulated for longer periods (4 hrs, 8hrs & overnight) with erythropoietin and SCF

Results in 4.2.2 had suggested that there may be differences in signalling between PV & controls with ≥2 hours cytokine stimulation. Twenty four samples which had been starved or stimulated for longer periods with Epo +/- SCF (4 hours, 8 hours & overnight) were therefore assessed to consider this issue. In order to control for proliferative differences over this longer time period, basal activity relative to total protein was expressed as a ratio of stimulated activity at each time point. Results are shown in Figure 15[4].

Figure 15[4]: Quantitative evaluation of the signalling activity of erythroid progenitors over a protracted time course

(erythroid progenitors were starved for 2 hrs, incubated plus/minus cytokines and paired basal & stimulated samples lysed after a further 4hrs, 8hrs & overnight incubation. Western blots were probed for pAKT, pGSK3, pERK & pSTAT5 and expression quantitated relative to total protein. The basal / stimulated ratio was calculated for each time point & average results by diagnosis shown from a total of 24 experiments including 14 PV, 8 IE & 2 normals from signals at 4hrs[n=10], 8hrs [n= 6] & overnight [ON, n= 8], standard error of the mean shown)



As these results have been expressed as a ratio in order to control for proliferative effects, it is not possible to say whether there was individual variation in basal or stimulated signalling between PV & controls. In addition these experiments include a mixture of those stimulated with Epo alone and those stimulated with Epo + SCF; unfortunately the sample size was not sufficient to allow separate analysis of these groups. Despite these limitations this evidence suggests that the elevated PI3K activity seen in PV is increased for sustained periods in erythroid progenitors derived from patients with PV. This sustained effect was not apparent for the MAPK or STAT pathways.

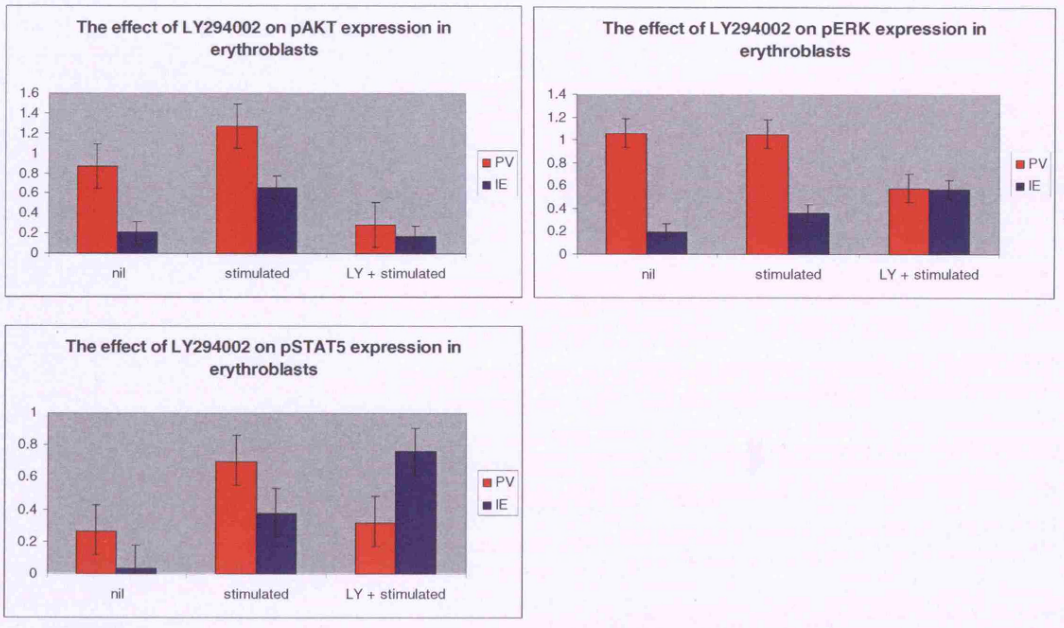
4.3 The effects of PI3K inhibition on aberrant signalling in PV erythroblasts

Having established differences in erythroid signalling in PV, further work went on to assess whether specific pathway inhibitors might have differential effects on PV erythroid progenitors.

4.3.1 Assessing the effects of PI3K inhibition with LY294002 in PV

Initial data had shown that inhibition of PI3K in erythroid progenitors reduced the phosphorylation of AKT and p70S6K but not of ERK or STAT5 (see Figure 5[4]). In order to investigate the effects of PI3K inhibition in potentially aberrant PV signalling, further experiments were done with 13 samples including 6 from patients with PV. Results are shown in Figure 16[4].

Figure 16[4]: The effects of PI3K inhibition on aberrant PV signalling
(western blots were made from 13 samples of erythroblasts (6 PV, 7 IE) starved -/+ stimulated with Epo (n=6) +/- SCF (n=7) -/+ pre-incubation with LY294002, blots were probed for for pAKT, pERK, pSTAT5 & total protein and relative quantitation is shown with standard error of the mean)



These results confirmed that LY294002 inhibits phosphorylation of AKT but not ERK or STAT5 in erythroid progenitors derived from patients with idiopathic erythrocytosis as is seen in normal samples.

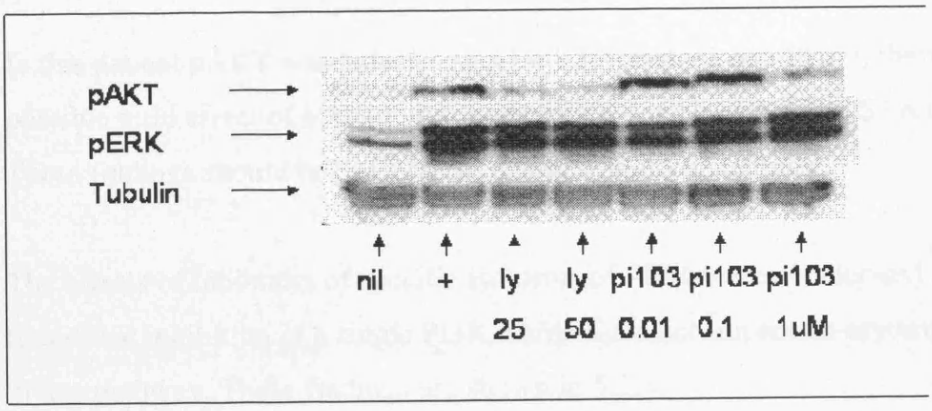
A different pattern emerged for samples from patients with Polycythaemia Vera: LY294002 reduced stimulated phosphorylation of AKT, ERK and STAT5 and, in the case of AKT and ERK, additionally reduced the increased basal activity noted in PV. In also appeared that LY294002 may have more profound effects on signalling in PV than in IE as, whilst the stimulated signal was significantly higher in PV, the inhibited signal was not. This was particularly evident for STAT5 phosphorylation.

4.3.2 The effects of novel PI3K inhibitors on signalling

Work from several groups has raised doubts about the selectivity of LY294002 and shown inhibitory effects on Pim-1 & protein kinase CK2 as well as PI3K signalling. Therefore, more recently developed novel PI3K inhibitors were evaluated. These experiments were unfortunately limited in number due to time constraints. TF1 cells (expressing wild type JAK2) were starved overnight then incubated with either LY294002 or PI103, a highly selective Class I PI3K inhibitor, [Raynaud et al, 2007] prior to stimulation with GM-CSF. The results are shown in Figure 17[4]. Similar effects were seen in Mo7e cells with PI103 inhibiting GM-CSF induced AKT phosphorylation at a concentration of 250nM.

Figure 17[4]: Comparing the effects of LY294002 with the Class 1 PI3K inhibitor, PI103 in tumour cells

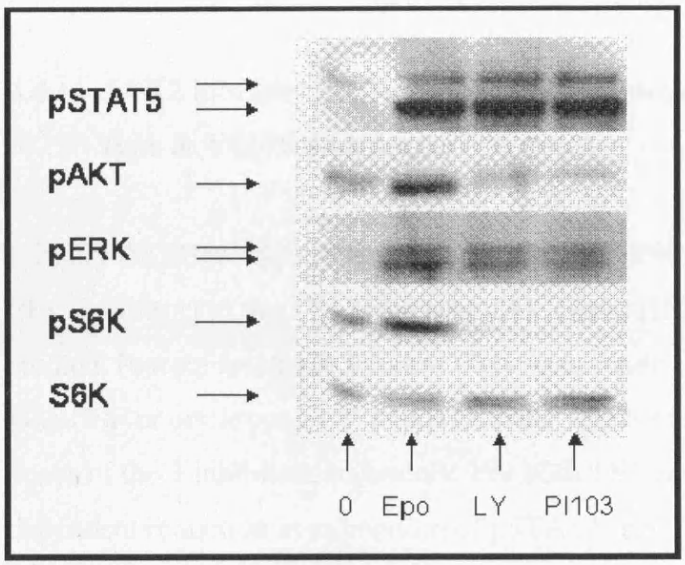
(western blots from protein lysates of TF1 cells starved then incubated for 1 hour at 37^0C with LY294002 [ly] at 25 or 50uM or PI103 [pi103] at 0.01, 0.1 or 1uM then stimulated with GM-CSF for 10 mins)



These results indicated that PI103 at a dose of 1uM inhibited PI3K but not MAPK in TF1 cells. Further studies were done to evaluate if PI103 would additionally affect the MAPK & STAT pathways in PV samples, as LY294002 had done. 3 experiments using erythroblasts from 1 normal CD34+ derived sample, 1 IE and 1 patient with PV were done. Findings in the 2 control subjects were as for those in the TF1 cells (the

MAPK & STAT pathways were unaffected). Results from the patient with PV are shown in Figure 18[4].

Figure 18[4]: Comparing the effects of PI3K inhibitors in PV erythroblasts (erythroblasts starved and stimulated with erythropoietin -/+ pre-incubation with either LY294002 [LY] or PI103, western blots probed for PI3K effects; pAKT & pS6K and for pSTAT5, pERK and S6K as total protein)



In this patient pAKT was reduced with both LY294002 and PI103, there was a possible mild effect of both inhibitors on pERK but no effect on pSTAT5 expression. These findings should be explored in a larger group of samples.

The effects of inhibitors of specific isoforms of PI3K were also looked at and it was found that inhibition of a single PI3K isoform did not impact on erythroid signalling in any pathway. These findings are shown in 5.2.4.

4.4 The effects of specific inhibitors of JAK2 on signalling

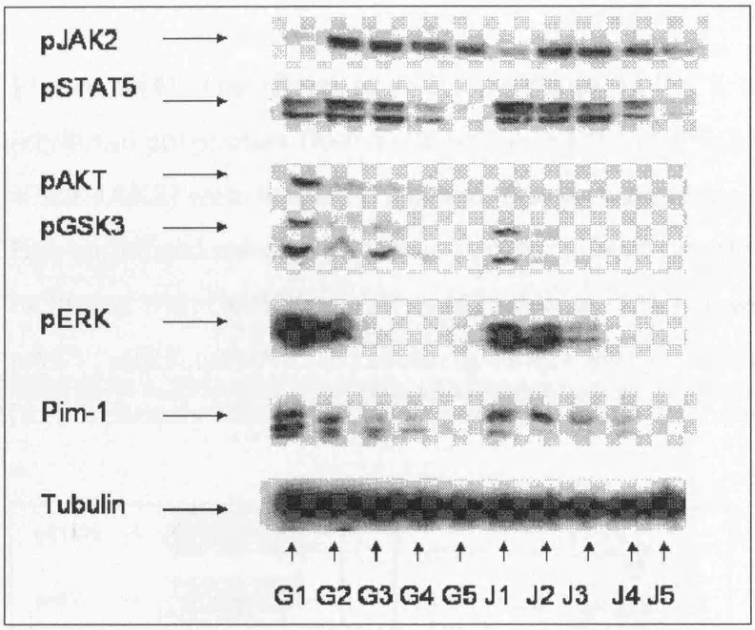
Since data was published on the high degree of association between V617F JAK2 and a diagnosis of PV, there has been considerable interest in the therapeutic potential of JAK2 inhibitors in this and other myeloproliferative disorders. This study explores the effects of 2 such inhibitors in haematopoietic cell lines expressing V617F and wild type JAK2 and then goes on to explore potential differential effects in primary PV erythroblasts.

4.4.1 JAK2 inhibitors & signalling in haematopoietic cell lines expressing wild type & V617F JAK2

Initially the effect of Go6976 (JAK2 & PKC inhibitor), Jak Inhibitor 1 and Go6983 (a PKC inhibitor) in the TF1 (wild type JAK2) and HEL (V617F JAK2) cell lines was studied. Protein lysates & western blots were made from TF1 cells (in the presence of GM-CSF or erythropoietin) and HEL cells which had been incubated with a range of doses of the 3 inhibitors separately. For both TF1 and HEL cells, there was dose-dependent reduction in expression of pSTAT5, pAKT, pGSK3, pERK and PIM-1 with Go6976 & Jak Inhibitor 1 but not with Go6983. A representative experiment using HEL cells is shown in Figure 19[4].

Figure 19[4]: The effect of the JAK2 inhibitors Go6976 and Jak Inhibitor 1 on signal transduction in haematopoietic cell lines

(representative western blots from protein lysates of HEL cells incubated with Go6976 [G1=50nM, G2=250nM, G3=500nM, G4=1uM, G5=2.5uM] & Jak Inhibitor 1 [J1=10nM, J2=100nM, J3=250nM, J4=0.5uM, J5=1uM], increased pJAK2 due to mode of action of inhibitor)



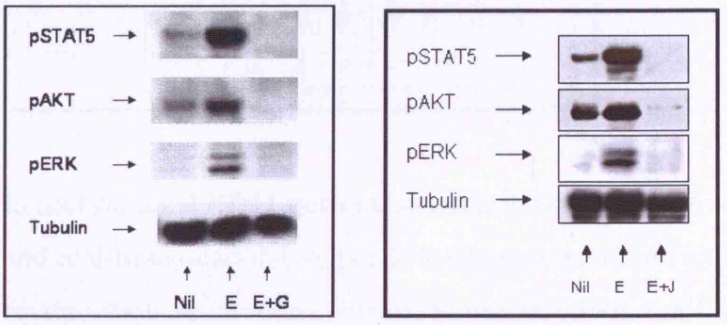
There was increased signal for pJAK2 with the JAK2 inhibitors. Our group previously reported this finding which is due to the mode of action of these and other Jak inhibitors in binding & stabilising phosphorylated JAK2. Under these conditions pJAK2 accumulates but is unable to activate downstream signalling. [Grandage et al, 2006] This finding illustrates that probing western blots for pJAK2 may not be a useful method in screening for potential JAK2 inhibitors.

4.4.2 The effects of specific inhibitors of JAK2 on erythroid signalling

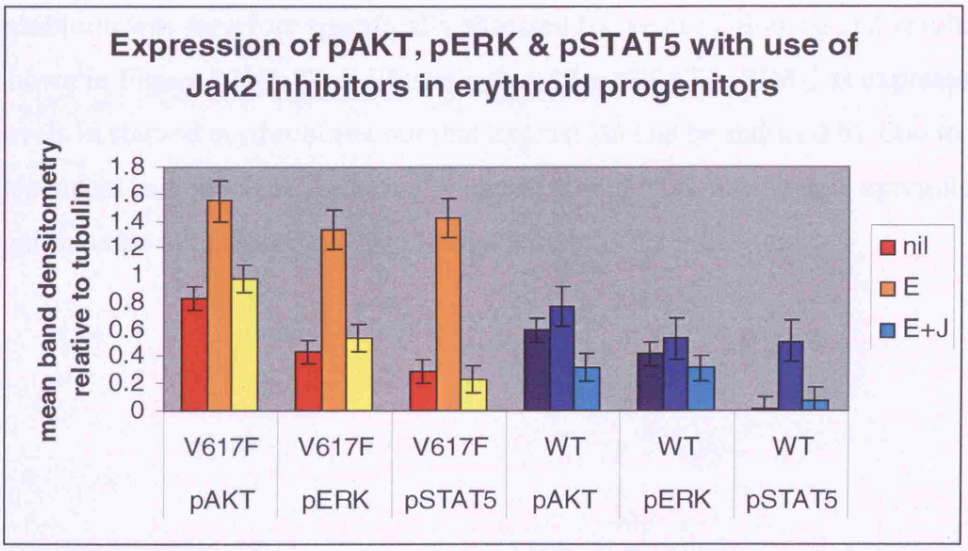
The effects of JAK2 inhibition on erythroid progenitors were investigated in 10 experiments including 5 samples from patients with PV & V617F JAK2. Mean results by JAK2 status from these experiments are shown in Figure 20[4]. These results show that JAK2 inhibition markedly reduces stimulated pSTAT5 in PV and control cells but also reduces stimulated pAKT and pERK in PV.

Figure 20[4]: The effects of JAK2 inhibition in PV & control erythroid cells (erythroid progenitors from 5 patients with PV [V617F JAK2] & 5 controls [wild type = WT JAK2] were washed & starved, lysates were made from a basal sample [nil], an Epo-stimulated sample [E] and an Epo-stimulated sample which had been pre-incubated with Go6976 [E+G] or Jak Inhibitor 1 [E+J], western blots were probed for pAKT, pERK, pSTAT5 & tubulin & results were quantitated, **a.** representative plots from 2 patients with PV, **b.** average results shown, standard error of the mean plotted)

a.

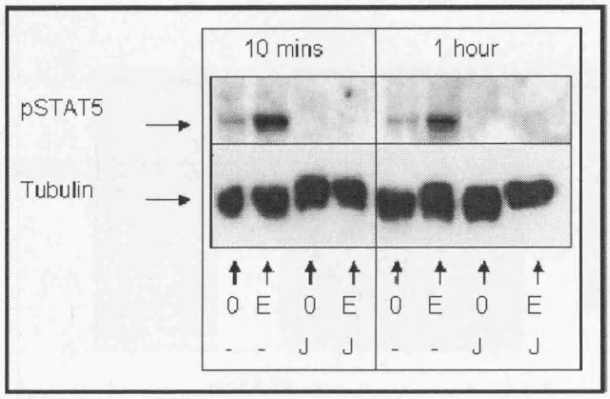


b.



Increased basal signalling was not apparent in all PV cases. Where there was an increase this was eliminated by Jak2 inhibition. This can be seen in Figure 20[4] and also in Figure 21[4]. This suggests that the increased constitutive signalling seen in PV is Jak2 dependent.

Figure 21[4]: Basal and stimulated pSTAT5 are reduced with JAK2 inhibition (Day 9 erythroblasts were washed and separated into 2 test groups for 10 mins or 1 hour epo stimulation time points; nil [0], epo [E], nil + Jak Inhibitor 1 [0/J], epo + Jak Inhibitor 1 [E/J]. Western blots were made, probed for pSTAT5 and Tubulin and scanned as shown below)

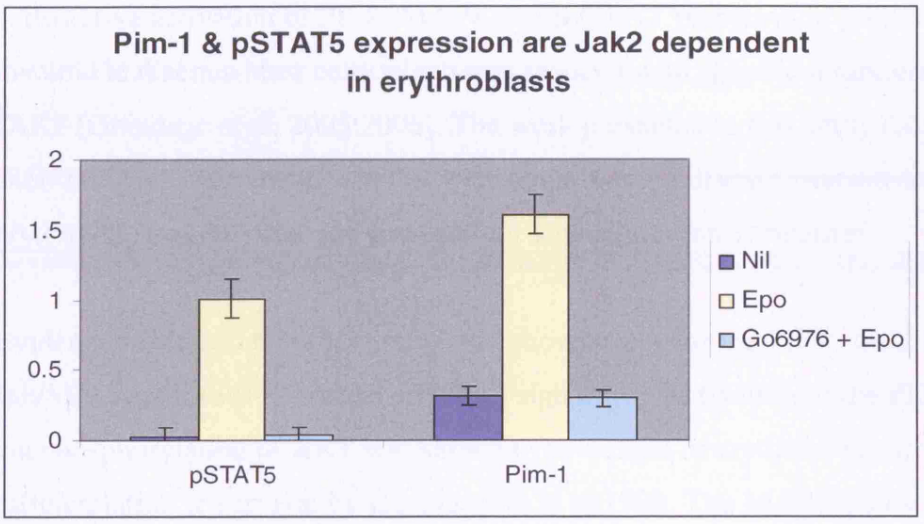


In section 6.2.2 PIM1 gene expression in erythroblasts is discussed as the microarray and real-time quantitative pcr data showed increased expression of PIM1 in PV erythroblasts relative to controls. Subsequently in 6.3.1 it is shown that PIM1 mRNA expression was reduced by inhibitors of JAK2. PIM-1 protein expression with JAK2 inhibition was therefore specifically assessed by western blotting and results are shown in Figure 22[4]. They illustrate that, like pSTAT5, PIM-1 is expressed at low levels in starved erythroblasts but that expression can be induced by Epo in a JAK2 dependent manner. It is additionally apparent that PIM-1 is basally upregulated in PV and that this upregulation is also reduced with JAK2 inhibition.

Figure 22[4]: JAK2 inhibition blocks Epo-induced Pim-1 expression in all erythroid progenitors and also basal upregulation in PV progenitors

(**a.**western blots were made from 5 samples of erythroblasts (2 PV [heterozygous V617F JAK2, 2 IE & 1 normal [wild type JAK2]) starved [0] -/+ stimulated with Epo [E] -/+ pre-incubation with Go6976 [G], blots were probed for for pSTAT5, Pim-1 & total protein and relative quantitation is shown with standard error of the mean. **b.** western blot from PV derived erythroblasts showing that both basal and stimulated PIM-1 are inhibited at 3 time points)

a.



b.

4.5 Discussion

Intracellular signal transduction is a highly complex dynamic process with individualized operating systems for each cell type which are adapted to the functional requirements of the cell. Our group initially studied erythoid progenitors derived from G-CSF mobilized stem cell collections & showed both that phosphorylation of signalling protein kinases was cytokine dependent in normal erythroid cells and also that PI3K signalling via GSK3 was critical to erythroid survival. [Somervaille et al, 2001]. Subsequent work by our group reported constitutive activation of PI3K, MAPK & Jak/STAT pathways in primary acute myeloid leukaemia blast cells which was reduced with specific inhibitors of PI3K and JAK2 [Grandage et al, 2005;2006]. The work presented in this study focuses on aberrant signal transduction in Polycythaemia Vera, a disease characterised by erythroid expansion with the potential for leukaemic transformation.

Evidence published by other groups has shown roles for the PI3K, MAPK & Jak/STAT pathways in normal erythroid signalling. Activation of the PI3K pathway via phosphorylation of AKT was shown to be critical to erythroid proliferation, differentiation & survival by Haseyama et al in 1999. The MAPK pathway which includes pRSK & pERK was also shown to synergise with the PI3K pathway to support erythropoiesis by Sui et al in 1998. Negative regulation of the Jak/STAT pathway by Suppressor of Cytokine Signalling 3 (SOCS-3) was shown to suppress erythropoietin induced proliferation by Sasaki et al in 2000.

Several cytokines have been shown to contribute to erythroid proliferation and survival but the 2 critical cytokines are Epo and SCF which act via their cognate receptors EpoR & c-Kit. Epo & SCF synergise to support erythropoiesis but activate signalling by different routes: EpoR lacks intrinsic protein tyrosine kinase activity & uses intracellular JAK2 for signal transduction. Epo signalling is therefore JAK2 dependent. By contrast, when SCF binds to c-KIT, which has intrinsic tyrosine kinase activity, the complex signals by creating binding sites for a variety of Src homology 2 domain-containing enzymes and adaptor proteins such as the p85 subunit of PI3K.

SCF signalling is therefore not directly JAK2 dependent [Munugalavadla & Kapur, 2005].

Epo, but not SCF, directly induces phosphorylation of STAT5 via JAK2 in erythroid progenitors. Epo also transiently induces phosphorylation of ERK in normal erythroid cells. SCF is the major contributor to phosphorylation of AKT (although Epo has some independent effect) in early erythroid progenitors but also sustains phosphorylation of ERK in these cells. [Arcasoy & Jiang, 2005]

This work initially focussed on the detailed position of GSK3 in haematopoietic cell lines and in primary erythroid progenitors comparing and contrasting findings. Firstly, SCF induced phosphorylation of GSK3 in Mo7e tumour cells was assessed. As expected, pAKT was reduced by the PI3K inhibitor, LY294002, but not by the MAPK inhibitor, U0126 (in fact U0126 increased pAKT). Phosphorylated ERK was reduced by U0126 but not by LY294002. Surprisingly, GSK3 phosphorylation did not directly mirror pAKT, but showed delayed kinetics with no effect of LY294002 apparent at a 10 minute stimulation time point but complete inhibition at a 4 hour time point. A similar pattern was observed in Mo7e cells, TF1 cells and 32 D cells. Where GSK3 phosphorylation was not inhibited by LY294002 alone, it was abrogated by the addition of U0126 to LY294002.The concentration of SCF used for stimulation in Mo7e cells was then reduced to more physiological levels & it was found that phosphorylation of GSK3 was PI3K dependent under these conditions. This observation contrasts with the immediate PI3K dependency of GSK3 apparent in stimulated erythroid progenitor cells at supraphysiological doses of cytokine stimulation.

One possible explanation for these findings was that the predominant PI3K dependency of GSK3 in erythroid progenitors might result from an inability of SCF to activate the MAPK axis in these cells. In order to address this, experiments were performed using erythroid progenitors & it was found that either Epo or SCF stimulation could induce MAPK phosphorylation. In addition, Tetradecanoyl phorbol-myristate acetate (TPA) has been shown to act via protein kinase C (PKC) to stimulate phosphorylation of ERK & GSK3 in neuronal cells [Medina et al, 2005]. In this study Mo7e cells and erythroid cells were stimulated with TPA and it was found

that GSK3 was also phosphorylated in these cells in a MAPK dependent fashion. These findings show that although GSK3 can be phosphorylated through the MAPK pathway in erythroid cells, SCF or Epo utilize PI3K for this purpose in these cells, despite activating MAPK.

In erythroid progenitors stimulated with Epo or SCF, it was confirmed that STAT5 phosphorylation was induced by Epo but not SCF and was PI3K and MAPK independent with short periods of stimulation. Using Mo7e cells this period of cytokine stimulation was extended and it was found that, whilst STAT5 activation was overwhelmingly PI3K & MAPK independent for short periods of stimulation, there was evidence of cross-talk between pathways with more protracted stimulation. This analysis was not pursued in erythroid progenitors.

The published literature on signalling regulation in Polycythaemia Vera erythroblasts is limited. Ugo et al [2004] introduced PI3K, JAK/STAT & ERK/MAPK inhibitors into liquid cultures of bone marrow derived erythroblasts and noted reduced Epo dependent terminal differentiation with all 3 inhibitors & reduction of Epo independent differentiation with 2/3 inhibitors (not MAPK). Zeuner et al [2006] observed that, in the presence of saturating levels of Epo, there was no difference between the PV and control signal for pAKT or pERK in peripheral blood derived erythroblasts. By contrast, they observed that when Epo was reduced or withdrawn, pAKT and pERK were maintained in PV erythroblasts but not in normal controls. Dai et al [2005] did not detect any constitutive activation of pAKT in PV but did note that Epo and SCF induction of PV erythroblasts resulted in increased pAKT expression which was sustained in 4/5 PV but not in controls. This group also looked at pGSK3 and reported that effects were similar to those seen with pAKT although their data shows that the kinetics of pGSK3 may be different to pAKT in PV.

This study set out to clearly establish signalling abnormalities in primary PV erythroid progenitors looking at each of the PI3K, MAPK & STAT pathways. All of the PV samples used in these experiments expressed V617F JAK2 and all of the controls expressed wild type JAK2. The optimal erythroid culture conditions were first established by building on the work of others. The signal in each pathway by day of in vitro culture was then looked at to assess pathway activity and Day 9 was

selected for further study. After 9 days of in vitro culture there was a homogeneous population of basophilic erythroblasts in sufficient number to study protein effects and each of the PI3K, MAPK & STAT pathways were active. All further experiments were performed on Day 9 erythroblasts to facilitate comparison between subject groups.

The effects on signal transduction of stimulation with SCF & Epo were initially assessed over a period of up to 2 hours in a small group of samples including 4 from patients with IE and 2 from patients with PV & homozygous expression of V617F. There was a suggestion of increased signalling with stimulation in all pathways in the PV samples.

The next step was to look at a larger group of 17 samples including 8 IE, 7 PV (3 homozygous, 4 heterozygous for V617F JAK2) & 2 normal controls. In these experiments the basal and short term (<1hr) stimulated effects with Epo & SCF were assessed. A ratio of these values was then derived in order to assess whether the maximal achievable signal might be greater than that simply induced by an increase in basal activity. Using this method there were clear differences between PV & IE in the basal, stimulated and basal / stimulated signal for the PI3K & MAPK pathways. In this group of studies pSTAT5 was only assessed in 4 of the PV patients and there was no overall difference between PV & controls in this small group.

In the knowledge that Epo transduction is Jak dependent & that SCF transduction is Jak independent, results from the previous group of experiments were contrasted with a further cohort of 16 samples including 7 with IE and 9 with PV (2 homozygous, 7 heterozygous for V617F JAK2) using Epo alone for stimulation. With this approach it was again apparent that there was increased basal & stimulated signal in the PI3K & MAPK pathways with no excess in the basal / stimulated signal for these pathways. In contrast to the previous results, when studying this larger group of PV subjects, there were clear increases in basal, stimulated and basal / stimulated signal for STAT5 (which is, as has been shown, SCF independent).

Overall, when the basal signal of pAKT, pERK & pSTAT5 between 18 patients with PV (& V617F JAK2) and 21 control subjects (all wild type JAK2) was compared,

there were highly significant increases for PV in all 3 pathways. The conclusion is therefore that, not only is there constitutive activation affecting each of the PI3K, MAPK & Jak/STAT pathways in PV but that the cytokine hypersensitivity of PV erythroblasts observed by other authors is in addition to, and not just as a result of, this constitutive basal activation.

Having noted an excess in the basal signal expressed as a proportion of the maximal signal for pAKT & pERK (Epo + SCF) & pSTAT5 (Epo) for stimulation periods of <1 hour in PV samples, further work went on to see if this effect was sustained for longer periods of stimulation. It was found that increased signalling in PV was sustained up to 4 hours for pSTAT5 and up to 24 hours for pAKT. Together these findings point to a critical role for PI3K signalling in PV.

Blockade of individual signalling pathways has been shown to affect erythroid progenitor function. LY294002 is a PI3K inhibitor which has been shown to reduce phosphorylation of AKT and reduce Epo & SCF supported proliferation and maturation of erythroblasts. U0126 has been shown to reduce phosphorylation of ERK and Epo-dependent viability in erythroblasts. [Myklebust et al, 2002] Absence of STAT5 in mice has been shown to reduce erythroblast survival & differentiation. [Sokolovsky et al, 2001].

Having identified this crucial role of PI3K in aberrant PV signalling, the effects of LY294002 on erythroblasts from 6 patients with PV and 7 patients with IE were compared. LY294002 reduced expression of stimulated pAKT in both subject groups as expected but it also reduced the increased basal phosphorylation of AKT in PV erythroblasts. In addition, LY294002 decreased pERK and pSTAT5 in PV erythroblasts, but had no effect in IE erythroblasts. As it became clear that LY294002 also had inhibitory effects on the Jak/STAT target PIM-1 and on CK2, further work moved to use of the pan-PI3K inhibitor PI103 rather than LY294002. Results showed that PI103 effects were similar to LY294002 in cell lines and in primary erythroid progenitors; however there was not sufficient time to test PI103 in more than 1 PV sample. Further work was done to test the effects of inhibitors of individual PI3K isoforms but it was found that these did not exert independent effects in cell lines or in primary erythroid cells.

Whether V617F JAK2 has a primary or secondary role in PV pathophysiology, use of a JAK2 inhibitor may have therapeutic potential. This study used 2 specific JAK2 inhibitors, Go6976 & Jak Inhibitor 1, to explore the effects of JAK2 inhibition on erythroid signal transduction. The effects of Go6976 & Jak Inhibitor 1 in cells expressing wild type JAK2 (TF1 cells) and V617F JAK2 (HEL cells) were first assessed. These effects were compared with those of the inhibitor Go6983 to control for the PKC inhibitory element of Go6976. Both of the JAK2 inhibitors induced dose dependent reduction of signal in the PI3K, MAPK & STAT pathways of both wild type & mutant JAK2 cells. Go6983 had no effect.

Eleven experiments were performed where JAK2 inhibitors were incubated with primary erythroblasts in the presence of Epo including 6 patients with PV (heterozygous V617F JAK2), 4 patients with IE & 1 normal sample from CD34+ mobilized erythroid progenitors (all wild type for JAK2). As expected, stimulated pSTAT5 was reduced in PV and control subjects with JAK2 inhibition. JAK2 inhibition also reduced stimulated pERK and pAKT in PV. PIM-1 expression was also assessed in some erythroblasts including 2 samples from patients with PV and effects were comparable to that seen with pSTAT5 in keeping with the recognized status of PIM-1 as a Jak/STAT target. In a number of these studies in PV it was apparent that basal activation of pSTAT5 & PIM-1 seen in PV was suppressed by inhibitors of JAK2.

In summary, work presented in this chapter shows that signalling in the PI3K, MAPK & STAT pathways is both constitutively upregulated and hypersensitive over and above this basal activity in PV. PI3K dysregulation has been shown to be aberrantly sustained in PV erythroblasts and a key role for PI3K inhibition in controlling basal signalling abnormalities has been identified. In addition it is shown that JAK2 inhibition of erythroid progenitors derived from patients with PV expressing V617F JAK2 can suppress aberrant signal transduction in all pathways.

CHAPTER 5 – RESULTS 3

The effects of small molecule inhibitors on erythropoiesis

5.1 The effects of JAK2 inhibition on erythroid colony formation & erythroblast viable cell number

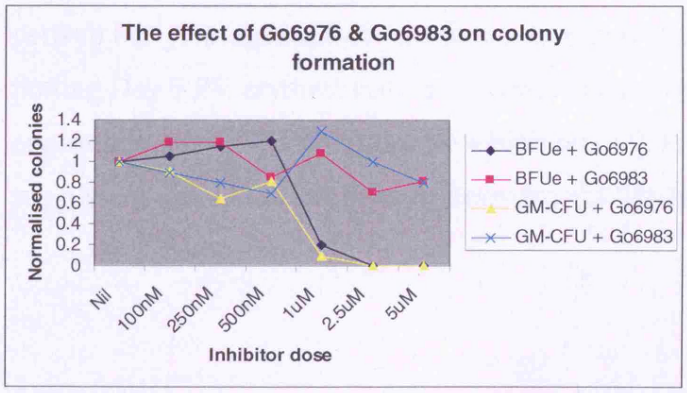
5.1.1 The effects of JAK2 inhibition on standard colony assays

Standard colony assays were set up using mononuclear cells derived from peripheral blood. Simultaneous colony assays were set up with either of the JAK2 inhibitors, Go6976 or Jak Inhibitor 1 as described in 2.3.3. Colonies were counted at Day 14 and the number of colonies in the absence of inhibitor was compared with the number in the presence of inhibitor.

Two initial experiments, using mononuclear cells from normal individuals, were performed to compare the effect on colony formation of Go6976 (a JAK2 & protein kinase C [PKC] inhibitor) with Go6983 (a PKC inhibitor) at concentrations between 100nM and 5uM. Go6976 reduced erythroid (BFUe/CFUe) & myelomonocytic (GM-CFU) colony formation, whereas Go6983 had no effect as shown in Figure 1[5].

Figure 1[5]: JAK2 inhibition with Go6976 reduces erythroid & myelomonocytic colony formation

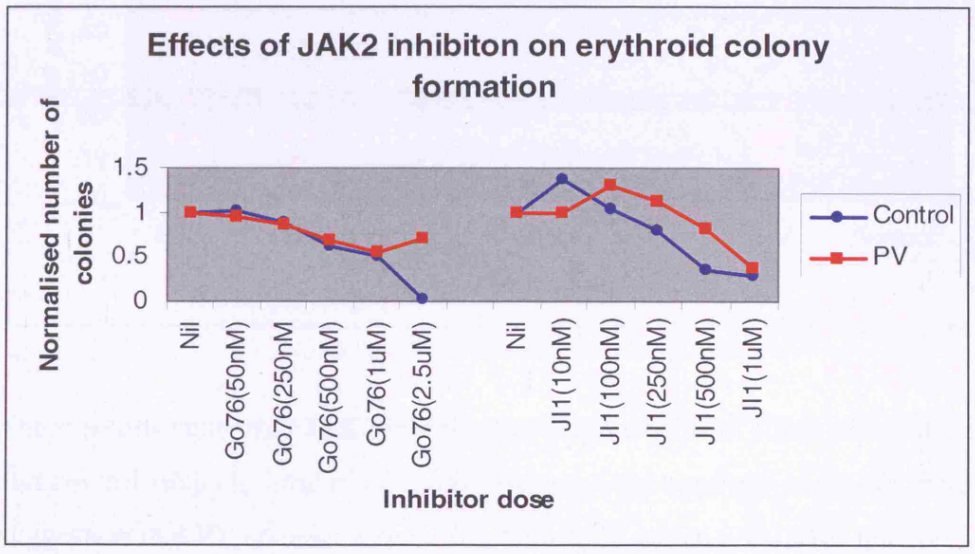
(results from 2 experiments comparing the effects on erythroid [BFUe] & myelomonocytic [GM-CFU] colony formation between JAK2 + PKC inhibition [Go6976] with PKC inhibition alone [Go6983]



A further 4 experiments were performed to determine comparable effective inhibitory doses of Go6976 and Jak Inhibitor 1 on erythroid colony formation. The mean of normalised values including material from 2 normal individuals, 1 with secondary polycythaemia and 1 with Polycythaemia Vera is shown in Figure 2[5].

Figure 2[5]: Comparing the effects of Go6976 and Jak Inhibitor 1 on erythroid colonies from 4 individuals

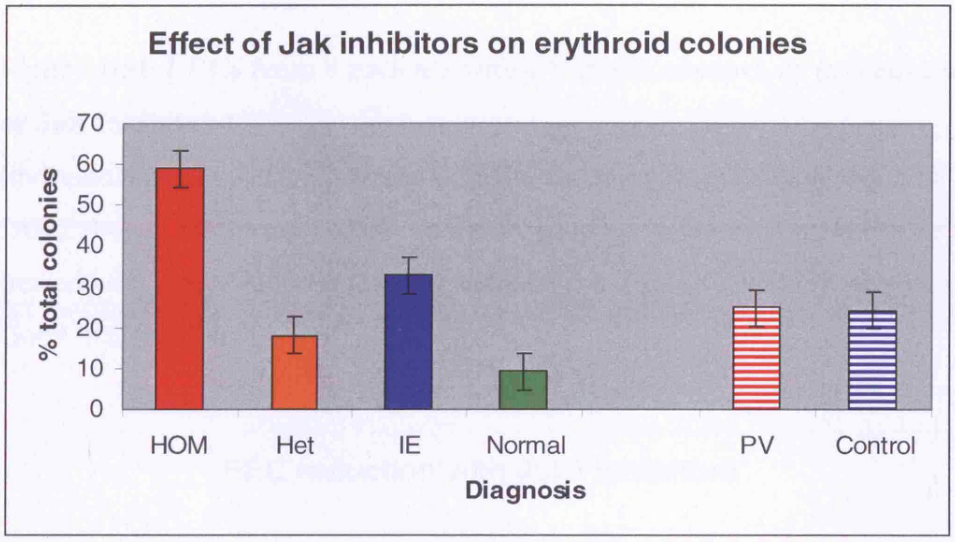
(erythroid colony numbers shown normalised to colonies in the absence of inhibitor, values shown for increasing doses of the JAK2 inhibitors Go6976 or Jak Inhibitor 1 [JI1], mean of 3 control samples shown with single PV sample, differences are not statistically significant)



Having established the dose responsiveness of standard colonies to JAK2 inhibitors, the effects of JAK2 inhibitors according to JAK2 status were assessed on a larger subject group. Forty colony assays with added Go6976 / Jak Inhibitor 1 were performed. Averaged results are shown in Figure 3[5]. A further single experiment putting Day 7 PV erythroblasts from liquid culture into colony assays resulted in an erythroid colony ratio (inhibitor:no inhibitor) of 0.46 with added Go6976 at 1uM suggesting that JAK2 inhibitory effects are not limited to early erythroblasts.

Figure 3[5]: Results from 40 colony assays with added inhibitors of JAK2 according to JAK2 status

(mean erythroid colony numbers with either added Go6976 or Jak Inhibitor 1 [JI1] shown as a percentage of total erythroid colonies without inhibitors by JAK2 status [n=number of JAK2 inhibited samples]; PV with homozygous V617F [HOM, n=4], PV with heterozygous V617F [Het, n=20], controls with wild type JAK2 [includes IE, n=10, Normal, n=6], calculated standard error of the mean shown)



These results show that JAK2 inhibition reduces erythroid colony formation in PV and control subjects similarly overall. Although the numbers are small there is a suggestion that PV samples expressing homozygous V617F JAK2 may be less sensitive to JAK2 inhibition.

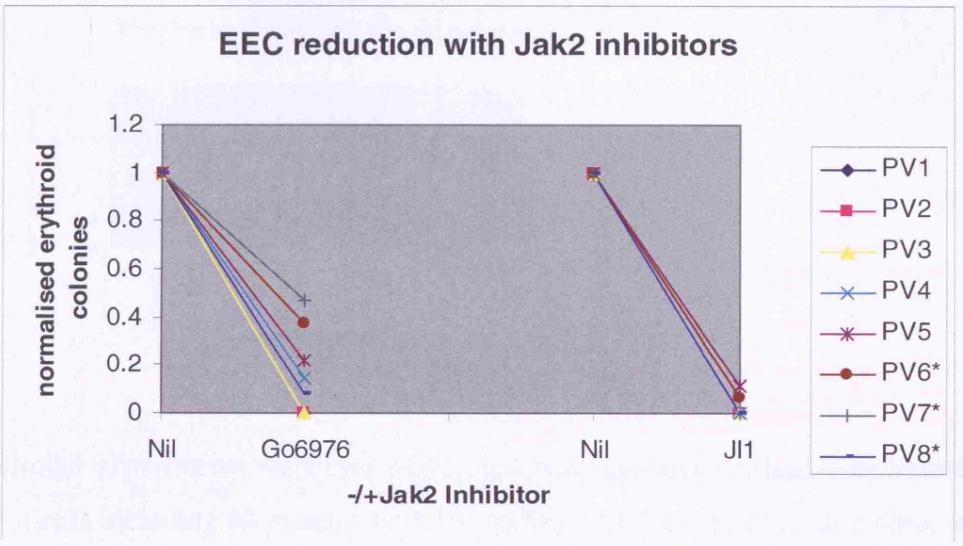
At the same time the effects of JAK2 inhibition on GM-CFU formation were assessed. In 22 assays Go6976 reduced GM-CFU to 0.80 +/- 0.11 & Jak Inhibitor 1 reduced GM-CFU to 0.25 +/- 0.07. There was no significant difference between PV patients and controls.

5.1.2 JAK2 inhibition of erythropoietin independent colonies

In 8 patients with PV and heterozygous expression of V617F JAK2, Go6976 or Jak Inhibitor 1 was introduced into EECs. Go6976 2.5uM reduced mean EECs to 0.07 (ratio to no inhibitor control) overall (n=5, PV1-5). Go6976 1uM reduced EECs to 0.31 (n=3, PV5-8*). Jak Inhibitor 1 at 1uM reduced mean EEC formation to 0.02. These results are shown in Figure 4[5] and confirm that JAK2 inhibition dramatically reduces the erythropoietin-independent colonies which are the hallmark of PV.

Figure 4[5]: EECs from 8 patients with PV in the absence or presence of Go6976 or Jak Inhibitor 1

(individual results from 8 patients with PV and heterozygous expression of V617F JAK2 shown, number of colonies normalised to no inhibitor control [nil], PV 1-5 treated with 2.5uM Go6976 & 1uM Jak inhibitor 1 [JI1], PV5*-8* treated with 1uM Go6976 & 1uM JI1)

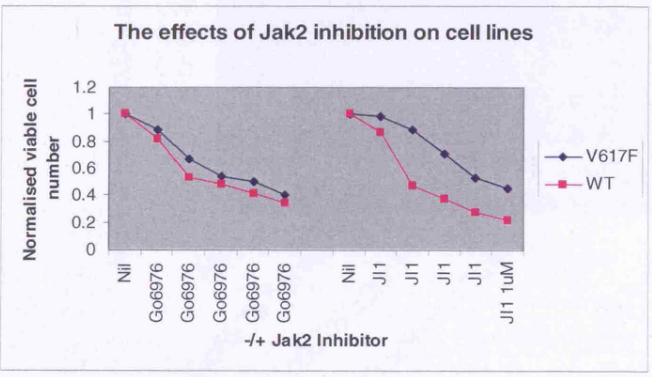


5.1.3 The effects of JAK2 inhibition on cell number in haematopoietic cell lines and primary erythroblasts

Go6976 and Jak Inhibitor 1 were introduced into 5 MTS assays using cells expressing mutant JAK2 (HEL) and 3 assays using cells expressing wild type JAK2 (Mo7e & TF1 with added cytokines). A clear dose response effect was apparent for both inhibitors and averaged results are shown in Figure 5[5] below.

Figure 5[5]: Results from 8 experiments assessing the viable cell number of tumour cells expressing V617F or wild type JAK2 in the presence of Go6976 or Jak Inhibitor 1

(averaged results from MTS assays where Go6976 or Jak Inhibitor 1 at concentrations of 50nM-2.5uM and 10nM to 1uM respectively were added at variable doses to HEL cells expressing V617F JAK2 [n=5] & Mo7e/TF1 cells with added GM-CSF expressing wild type JAK2 [WT, n=3]

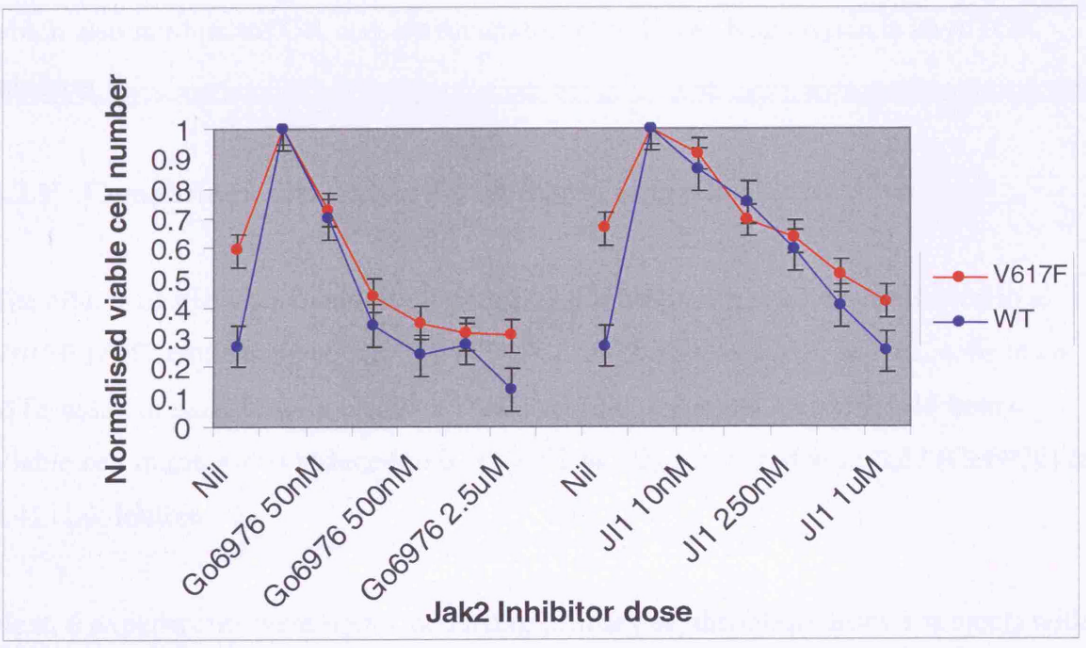


Similar experiments were then performed using primary erythroblasts from 20 patients including 10 patients with PV adding JAK2 inhibitors in the presence of Epo. The first key point to note is that JAK2 inhibition reduced the increased basal viable cell number seen in V617F mutant erythroblasts. Dose responsiveness was apparent for 2 distinct inhibitors. The dose response effects of inhibitors were equivalent between subject groups at lower doses but separation was apparent at the highest dose of both inhibitors as shown in Figure 6[5]a. At this higher dose there was a suggestion that homozygotes for V617F JAK2 may be less sensitive to JAK2 inhibition although numbers were small (Figure 6[5]b).

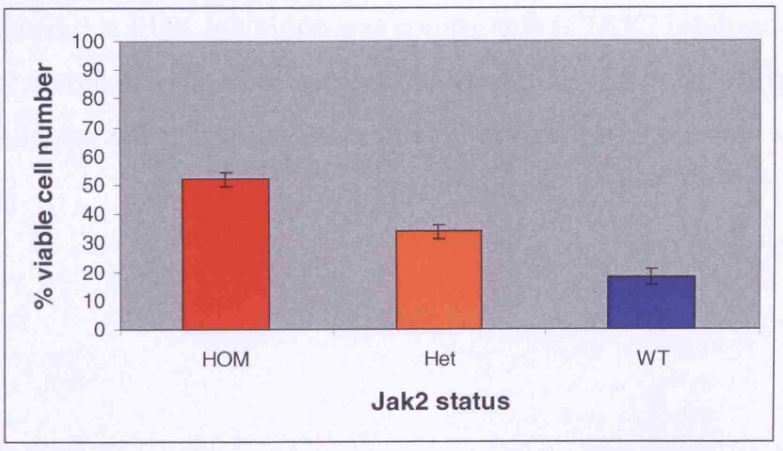
Figure 6[5]: Results from 40 experiments assessing the viable cell number of primary erythroid cells expressing V617F or wild type JAK2 in the presence of Go6976 or Jak Inhibitor 1

(**a.** Day 9 erythroblasts from 10 PV patients expressing V617F JAK2, 5 IE patients & 5 normals [all expressing wild type JAK2 = WT], cells were starved of cytokines [nil] or stimulated with erythropoietin -/+ increasing doses of 2 inhibitors of JAK2, Go6976 & Jak inhibitor 1 [JI1], mean values normalised to the erythropoietin only control are shown with calculated standard error of the mean, **b.** results separated by JAK2 expression for the highest dose of JAK2 inhibitors and expressed as a percentage of uninhibited viable cell number, HOM = homozygous V617F [n=4], Het = heterozygous V617F, [n=16,] WT = wild type JAK2 [n=20])

a.



b.



5.2 The effects of PI3 kinase pathway blockade on erythroid colony formation & erythroblast viable cell number

Work presented in the previous chapter showed that The PI3K pathway is activated in Polycythaemia Vera. This upregulation was found to be JAK2 dependent and further work therefore went on to look at whether PI3K blockade might be an alternative strategy for PV by assessing the effects of PI3K inhibition on erythroblast viable cell number and erythroid colony growth.

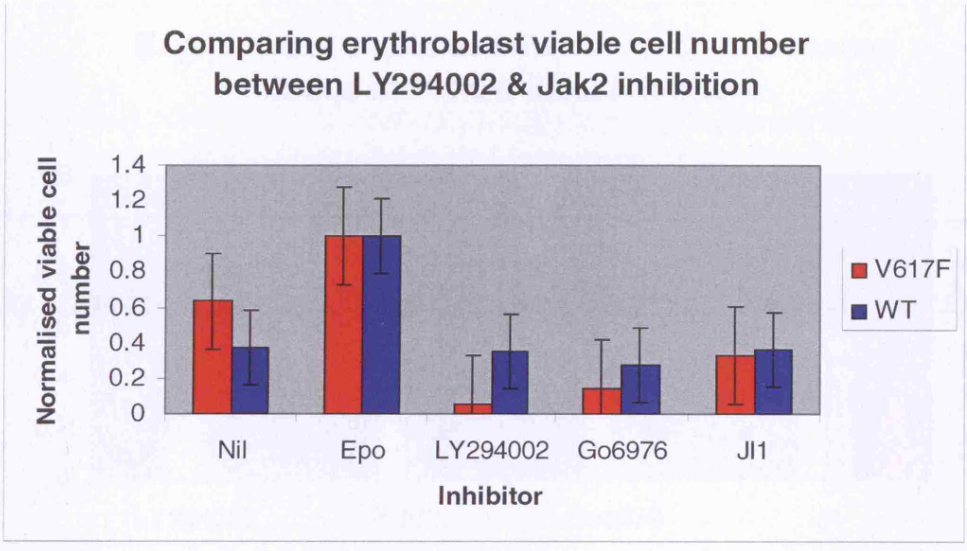
In order to explore the effects of PI3K inhibition 3 distinct inhibitors were initially used: LY294002 is a pan-PI3K inhibitor which is now known to inhibit PIM-1 and other serine/threonine kinases. PI103 is a highly selective Class I PI3K inhibitor which also inhibits mTOR, a downstream target of PI3K. Rapamycin is an mTOR inhibitor.

5.2.1 Comparing PI3K and JAK2 inhibition of erythroblasts

The effects of PI3 kinase inhibition with JAK2 inhibition were first contrasted in a V617F JAK2 expressing cell line; LY294002 at 50uM was added to HEL cells in an MTS assay in parallel with Go6976 2.5uM and Jak Inhibitor 1 1uM for 48 hours. Viable cell number was reduced to 0.10 (LY294002) compared with 0.37 (Go6976) & 0.42 (Jak Inhibitor 1).

Next, 6 experiments were performed using primary erythroblasts from 3 subjects with PV expressing V617F JAK2 and 3 samples which were wild type for JAK2. It was found that PI3K inhibition was comparable to JAK2 inhibition in reduction of erythroblast viable cell number. There was no significant difference between PV subjects and controls. Mean values of normalised results are shown in Figure 7[5].

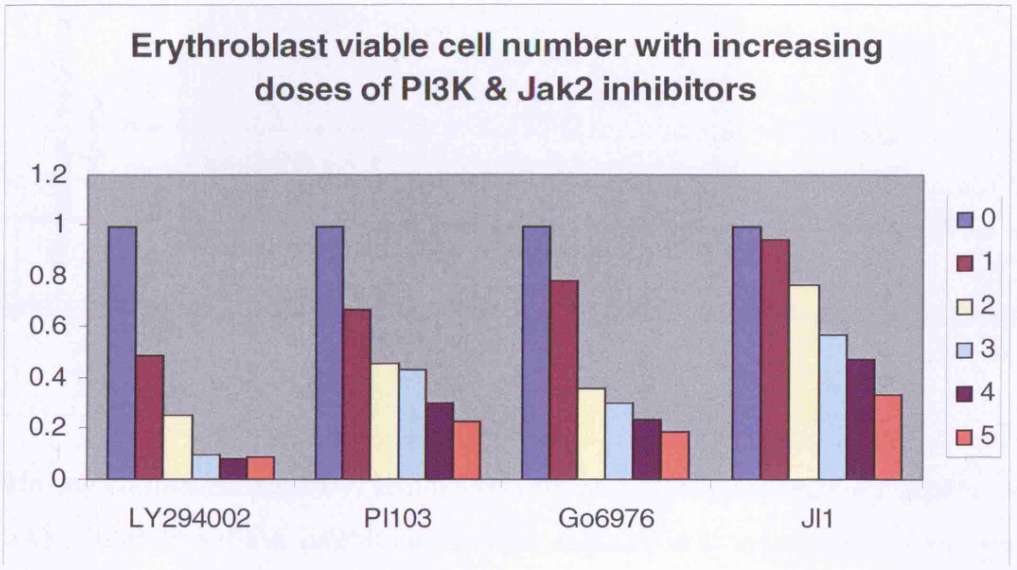
Figure 7[5]: Results from 6 experiments comparing the effects of PI3 kinase & JAK2 inhibition on erythroblast viable cell number using MTS assays
(samples include 3 from patients with PV expressing V617F JAK2, 2 IE & 1 normal all expressing wild type JAK2 [WT], mean erythroblast viable cell number as assessed by MTS assay after overnight incubation with or without the inhibitors LY294002, Go6976 & Jak Inhibitor 1 [JI1] shown, mean values normalised to no inhibitor Epo-replete wells shown, standard error of the mean plotted)



In order to confirm that this effect was due to PI3K (and not just Pim-1) inhibition, the effects of PI3K inhibition with LY294002 or PI103 were contrasted with JAK2 inhibition with Go6976 or Jak Inhibitor 1. Normal erythroblasts were incubated with PI3K or JAK2 inhibitors in the presence of erythropoietin. The results are shown in Figure 8[5] and illustrate the comparable effects of PI3K and JAK2 inhibition on erythroblast survival. A combination of PI3K & JAK2 inhibition was not tested as each inhibitor on its own critically reduced viable cell number.

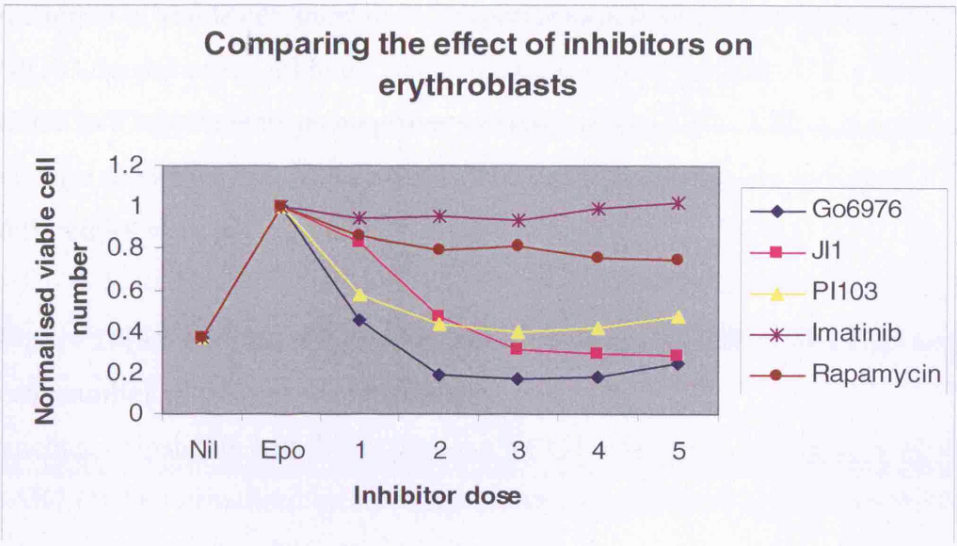
Figure 8[5]: Comparing the effects of PI3K inhibition with JAK2 inhibition on normal erythroblasts in liquid culture

(erythroblasts were washed and incubated in serum free medium overnight with no added cytokine [0] or erythropoietin and increasing doses of inhibitors [1-5], doses were 3/6/12/25/50um for LY294002, 10/100/250/500/1000nM for PI103, 50/250/500/1000/2500nM for Go6976 & 10/100/250/500/1000nM for Jak Inhibitor 1 [JI1], normalised viable cell number shown)



A further experiment went on to confirm this effect in erythroblasts derived from a patient with PV. On this occasion PI103 or PI3K inhibition was used together with the mTOR inhibitor, Rapamycin, and the effects of inhibition with the ABL & KIT kinase inhibitor, Imatinib were tested. The results are shown in Figure 9[5].

Figure 9[5]: MTS assay comparing PI3K and JAK2 inhibition in erythroblasts (results from a single experiment using erythroblasts from a patient with PV and increasing doses of 5 inhibitors [maximum doses were 2.5uM Go6976, 1uM Jak Inhibitor 1 [JI1], 1uM PI103, 1mM Imatinib & 20nM Rapamycin]



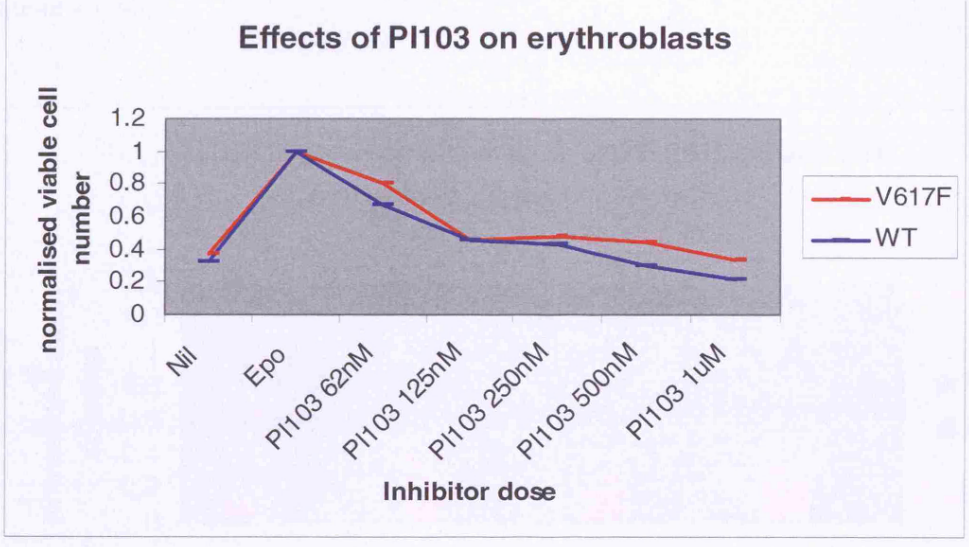
Having confirmed that PI3K inhibition reduced erythroblast viability similarly to JAK2 inhibition, PI3K inhibition was then assessed in a larger group of samples.

5.2.2 The effects of PI3 kinase inhibitors on erythroblast cell number in liquid culture

The dose response effect of the PI3 kinase inhibitor, PI103, was first tested on HEL cells at concentrations of 62nM – 1uM for 48 hours. There was dose dependent reduction in viable cell number in 2 separate experiments with mean reduction in (PI103 treated cells / inhibitor free cells) ratio to 0.41 at 1uM. This effect was then tested in 7 experiments using primary erythroblasts (5 PV, 1 IE, 1 normal) giving an average reduction of 0.30 with 1uM PI103. Results are shown in Figure 10[5], differences were not significant.

Figure 10[5]: Results of 7 experiments exploring the effect of PI103 on the viable cell number of primary erythroblasts

(includes 5 patients with PV expressing V617F JAK2 and 2 subjects with wild type JAK2 [WT], normalised mean values are shown, differences between the subject groups were not significant)



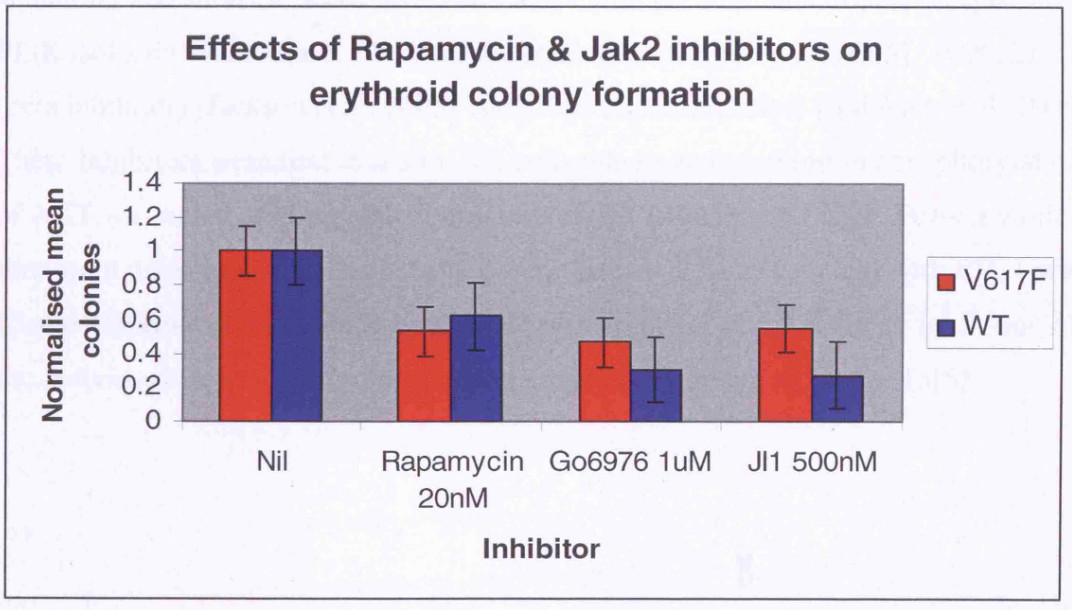
Rapamycin was introduced into 2 MTS assays using primary erythroblasts from patients with PV expressing V617F JAK2. Rapamycin 20nM reduced viable cell number relative to the no inhibitor control to 0.75 & 0.59 in the presence of erythropoietin. Viable cell number was reduced from 0.56 to 0.31 for erythroblasts incubated in the absence of erythropoietin.

5.2.3 The effects of PI103 & Rapamycin on standard colony assays and EECs

PI103 1uM was introduced into erythropoietin independent colonies (EECs) from a patient with PV in parallel with Go6976 2.5uM and Jak Inhibitor 1 1uM. EECs were completely abolished with PI103, Go6976 & Jak Inhibitor 1.

Rapamycin was introduced at 20nM into standard colony assays in 8 patients (4 PV & 4 IE) and the effects were compared with 1uM Go6976 1uM and 500nM Jak Inhibitor 1 and an inhibitor free control. Mean values from these experiments are shown in Figure 11[5] suggesting that Rapamycin has comparable inhibitory effects with JAK2 inhibitors. In an experiment in which Rapamycin was introduced into colony assays from Day 7 erythroblasts the erythroid colony ratio was 0.36. If this was reproducible it may suggest that the effects of Rapamycin are greater in less mature erythroblasts.

Figure 11[5]: Comparing erythroid colonies between 4 patients with PV (& V617F JAK2) and 4 with IE (& wild type JAK2)
(inhibitors added to standard colony assays, JI1 = Jak Inhibitor 1, standard error of the mean shown)

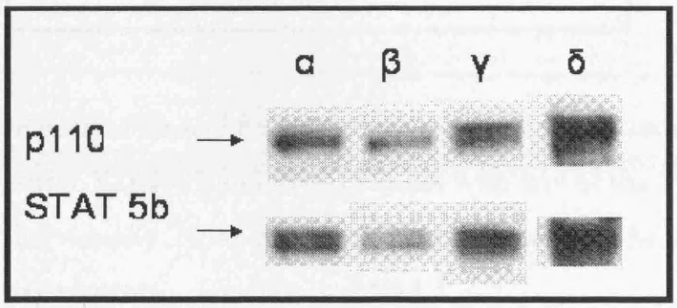


5.2.4 Exploring the effects of inhibitors of PI3 kinase isoforms

Having first established that the P110α, β and δ isoforms of PI3K were expressed in TF1, HEL and other cell lines, expression of these isoforms were then confirmed in erythroblasts. These results are shown in Figure 12[5].

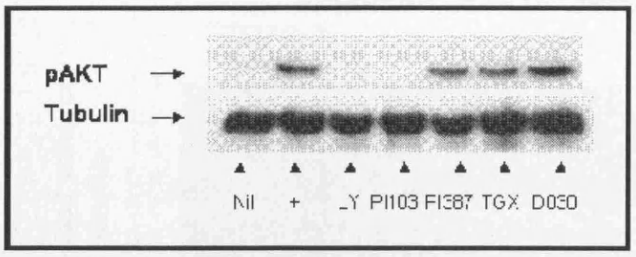
Figure 12[5]: Expression of the alpha (α), beta (β), gamma (γ) and delta (γ) isoforms in erythroblasts

(western blot of erythroblast protein lysates which were probed separately for each of the 4 PI3K isoforms and then for total protein)



In order to assess the differential contribution of PI3K isoforms to erythroblast PI3K signalling and survival, some novel inhibitors with predominant effects on specific PI3K isoforms were tested; PI387 (alpha inhibitor) [Knight et al, 2006], TGX221 (beta inhibitor) [Jackson et al, 2005] and D030 (delta inhibitor) [Sujobert et al, 2005]. These inhibitors were first tested in TF1 cells where no reduction in phosphorylation of AKT was noted despite reduction with both LY294002 and PI103. Further work then went on to assess affects in primary erythroblasts from 1 patient with PV, 1 with IE and 1 normal control. Once again there was no effect on pAKT with inhibition of the individual isoforms and representative results are shown in Figure 13[5].

Figure 13[5]: Pan-PI3K inhibitors reduce stimulated phosphorylation of AKT in erythroblasts but specific inhibitors of individual PI3K isoforms do not
(western blot shown depicting phosphorylated AKT and Tubulin expression in erythroblasts starved [nil] or stimulated [+] having been pre-incubated with a range of PI3K inhibitors – LY294002 [LY] & PI103 [pan-PI3K inhibitors], PI387 [α inhibitor], TGX-221 [β inhibitor] and D030 [δ inhibitor])



In each of these 3 experiments there was a clear reduction in phosphorylation of AKT with LY294002 and PI103 but not with any of the PI3K isoform inhibitors individually. None of the inhibitors affected the MAPK and STAT axes in these experiments.

Next, erythroblast survival with these inhibitors was studied. Viable cell number was assessed by MTS assay in 4 experiments using erythroblasts (2 PV, 2 IE) in the presence of erythropoietin [Epo, n = 4], stem cell factor [SCF, n = 1 (IE)] or insulin growth factor-1 [IGF-1, n = 1 (IE)]. IGF-1 alone did not sustain adequate cellular viable cell number to allow assessment of inhibitor effect. The effects of inhibitors on erythropoietin stimulated cells are shown in Figure 14[5]. The relative effects of Epo and SCF stimulated cells in 1 individual are shown in Figure 15[5]. These findings suggest that the reduced cytokine-dependent survival of erythroblasts seen with PI3 kinase inhibition is dependent on blockade of all isoforms simultaneously.

Figure 14[5]: 4 experiments assessing the effect of specific PI3 kinase inhibitors on erythroblast viable cell number

(mean normalised results from MTS assays in 4 individuals; 2 with IE expressing wild type JAK2 and 2 with PV expressing V617F JAK2, standard error of the mean shown)

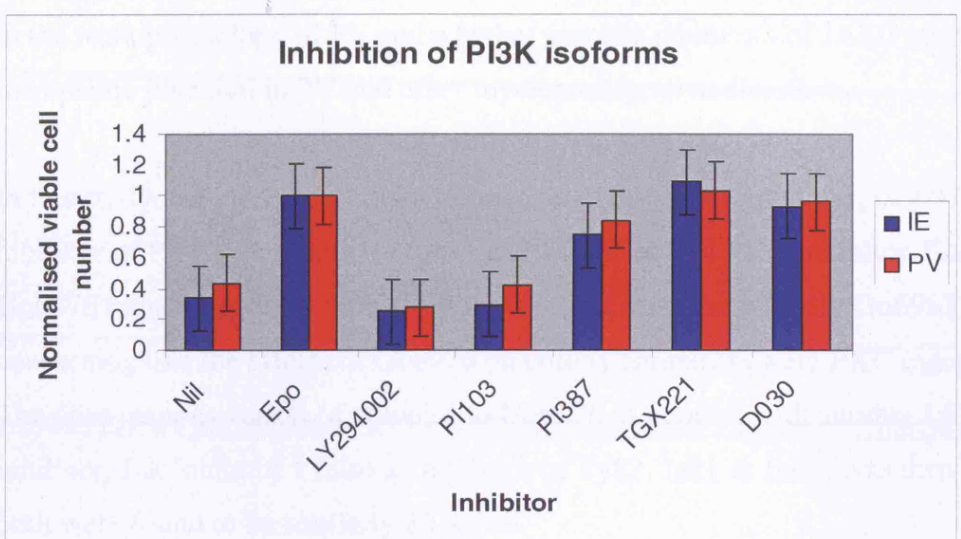
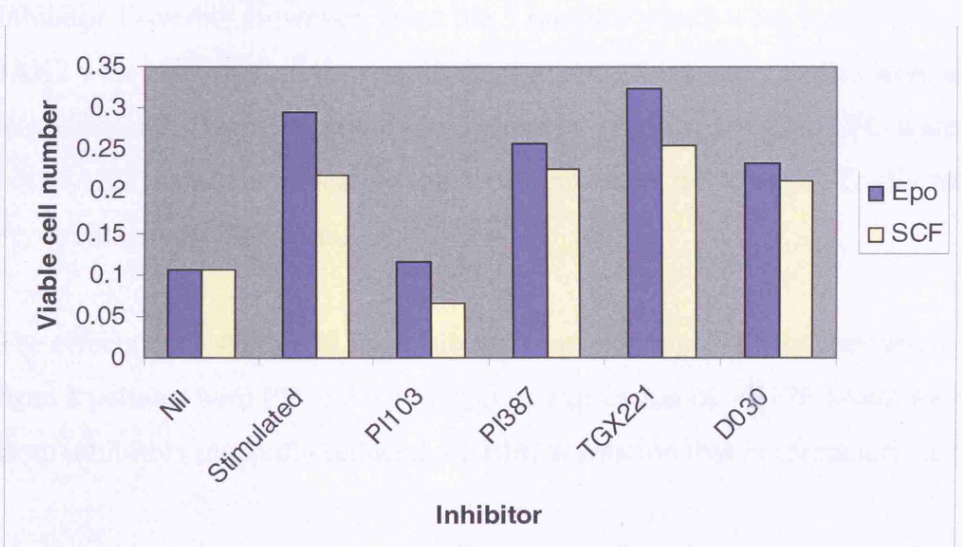


Figure 15[5]: The comparative effects of PI3K isoform inhibitors on erythroblasts stimulated with erythropoietin (Epo) or stem cell factor (SCF)

(both cytokines tested in parallel on D9 erythroblasts derived from a patient with IE and wild type JAK2, actual variations in viable cell number shown)



5.3 Discussion

It is now well established that a specific point mutation in JAK2 (V617F) is highly correlated with a diagnosis of Polycythaemia Vera. [James et al, 2005; Kralovics et al, 2005; Levine et al, 2005; Baxter et al, 2005; Zhao et al, 2005; Jones et al, 2005] Initial and subsequent research has gone on to look at how this mutation might be implicated in the pathophysiology of PV and whether specific inhibitors of JAK2 might have therapeutic potential in PV and other myeloproliferative disorders.

In this study, the effects on colony formation of the JAK2 inhibitor, Go6976 (also an inhibitor of PKC) were initially compared with a known PKC inhibitor, Go6983. Go6976 reduced erythroid and myeloid colony formation whereas Go6983 did not confirming that the effects of Go6976 on colony formation were PKC independent. The dose responsiveness of colonies to Go6976 in parallel with another JAK2 inhibitor, Jak Inhibitor 1 (also an inhibitor of Tyk2, Jak1 & Jak3) was then tested and both were found to be similarly effective.

Iniitial work explored whether there was a difference in effect on erythroid colony formation of the JAK2 inhibitors between patients with PV expressing V617F JAK2 and wild type JAK2 control subjects. Forty colony assays including 24 from samples expressing V617F JAK2 were performed and comparison by JAK2 status showed no significant difference in the number of erythroid colonies for either Go6976 or Jak Inhibitor 1 overall. However, when the 3 samples which were homozygous for V617F JAK2 were compared to the rest, it was apparent that these samples were significantly less affected by Go6976. In the same group of experiments GM-CFU were reduced by both JAK2 inhibitors indicating that these inhibitors did not specifically target erythroid colony formation.

The effects of Go6976 and Jak Inhibitor 1 on erythropoietin independent colonies from 8 patients with PV and heterozygous expression of V617F JAK2 were assessed. Both inhibitors markedly reduced the EEC formation that is characteristic of PV.

Evaluation of semi-solid colony assays is somewhat subjective in that colonies are counted manually. It is also difficult to precisely control cell density in a viscous medium. Previous experiments had shown that Day 9 erythroblasts retained functional differences between PV and controls and that these cells were still sensitive to JAK2 inhibition. Further experiments were therefore performed assessing the effects of JAK2 inhibitors on erythropoiesis by comparing the viable cell number of erythroblasts between V617F JAK2 and wild type control samples using MTS assays.

The principle was first tested in 8 experiments using haematopoietic cell lines expressing either V617F or wild type JAK2 in liquid culture. Dose-responsiveness in viable cell number with use of both Go6976 and Jak Inhibitor 1 was found. There was a trend to reduced sensitivity of cells expressing mutant JAK2 which did not reach significance.

A further 40 experiments were then performed using primary erythroblasts. Cells were washed free of residual cytokines and then incubated with no added cytokine or with added erythropoietin -/+ Go6976 or Jak Inhibitor 1 at a range of doses. There was a trend to reduced susceptibility of V617F erythroblasts to JAK2 inhibition which reached significance at higher inhibitor doses. Analysis of the small proportion of samples from patients expressing homozygous V617F (n=4) again suggested that these patients may be less sensitive to JAK2 inhibition than controls.

In 4.4.2 data was presented illustrating that increased basal signalling was seen in PV and that this was abolished with JAK2 inhibition. The experiments represented in 5.1.3 and discussed above showed that viable cell number in the absence of erythropoietin was increased in PV and that this increased basal viable cell number was suppressed with JAK2 inhibition. The implication is therefore that it is the JAK2 signal which accounts for the increased survival signalling seen in PV erythroid progenitors.

The data presented in this study showed that JAK2 inhibitors do reduce erythroid colony formation and erythroblast viable cell number but that they also affect myeloid colony formation. Using these 2 inhibitors primary erythroid cell survival is significantly reduced in V617F and wild type JAK2 samples although the inhibitory

effect may be lower in mutant cells. Ideally a JAK2 inhibitor with therapeutic potential might preferentially affect V617F JAK2 expressing erythroid progenitors.

The role of PI3K signalling in survival of diverse normal and malignant cells is now well established. Work done by our and other groups which is discussed in Chapter 4 showed that PI3K signalling is upregulated in PV erythroblasts and that this signal could be reduced with PI3K inhibitors as well as with JAK2 inhibitors. Further experiments were performed to assess whether this alteration in signalling with PI3K inhibitors was associated with a differential effect on PV erythroid progenitor viability.

Our group and others have used the Pan-PI3K inhibitor, LY294002, to explore the effects of PI3K signalling. Latterly it has become clear that LY294002 has broad effects on serine/threonine kinases including inhibition of the Jak/STAT target Pim1. [Jacobs et al, 2005] In view of the particular relevance of aberrant Jak/STAT signalling and its potential impact on erythroblast survival in PV, the highly selective Class I PI3K inhibitor PI103 (which also inhibits mTOR, a downstream target of PI3K) and the mTOR inhibitor, Rapamycin, were used in these experiments as well as LY294002.

The effects on primary erythroblast viable cell number of LY294002 were compared with Go6976 and Jak Inhibitor 1 in 6 primary samples, including 3 from patients with PV. Whilst the JAK2 inhibitors did not show different effects on the PV and control groups, there was a suggestion that LY294002 might be more effective in PV. This may relate to the additional inhibitory effects of LY294002 on Pim-1 as further experiments showed that PI103 and Rapamycin did not have greater effects on erythroblast viable cell number than JAK2 inhibitors in subsequent experiments.

PI3K inhibition was then looked at in a larger group. Firstly a dose dependent reduction in viable cell number of the V617F JAK2 expressing HEL cells with PI103 was observed. It was then found that the effect of PI3K inhibition with LY294002 was comparable to the effect of JAK2 inhibition in these cells. The effect of PI103 on primary erythroblast viable cell number was then assessed in 7 experiments including 5 from patients with PV. It was found that primary cells, including those from patients

with PV, showed dose-dependent reduction in viable cell number with PI103. It was additionally found that Rapamycin reduced PV erythroblast viable cell number in the presence or absence of erythropoietin.

Colony assays were performed using mononuclear cells derived from peripheral blood and it was found that PI103 could reduce erythroid colony formation and abolish EECs in PV. Colony formation in the presence of PI3K inhibitors was then contrasted between 4 patients with PV and 4 control subjects using Rapamycin and it was found that Rapamycin reduced erythroid colonies by approximately 50%. There was no significant difference in effect between PV and controls.

Having established that each of the α, β and δ isoforms of PI3K were expressed in primary erythroblasts, the effects of the novel PI3K isoform inhibitors, PI387, TGX221 & D030 were then assessed individually in these cells in comparison with LY294002 & PI103. It was found that inhibition of a single isoform had no effect on phosphorylation of AKT in contrast to the pan-PI3K blockade seen with LY294002 or PI103. Erythroblast survival was then assessed in 4 experiments using erythroblasts from 2 patients with PV and 2 control subjects. Whilst both LY294002 & PI103 reduced erythropoietin and SCF dependent viable cell number, the individual PI3K isoform inhibitors did not. The implication of these findings is that PI3K dependent survival in erythroblasts is not dependent on inhibition of a single isoform but on inhibition of all isoforms simultaneously.

The conclusion from these findings is that PI3K inhibition, or blockade of its downstream target, mTOR, reduces erythroid colony formation & primary erythroblast viable cell number in both PV and control subjects to levels comparable with JAK2 inhibitors. PI3K inhibitors might offer an alternative therapeutic approach in PV but inhibitors may need to block all PI3K isoforms simultaneously. An additional approach may be to explore the therapeutic potential of Rapamycin in PV.

CHAPTER 6 – RESULTS 4

The molecular imprint of Polycythaemia Vera

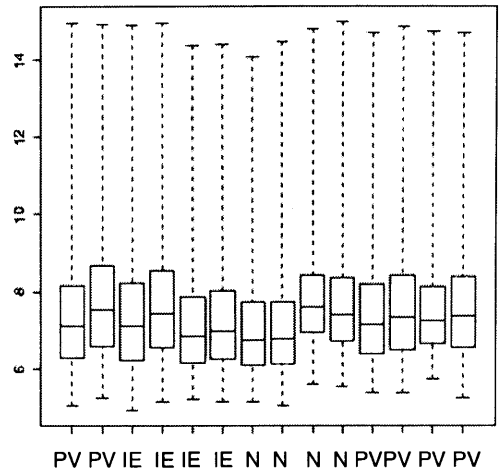
6.1 Affymetrix GeneChip expression profiling of erythroblasts From patients with PV and control groups

6.1.1 Quality control

In order to derive sufficient high quality erythroblast RNA suitable for microarray analysis, over 30 separate primary patient venesection packs were processed in an identical fashion. The 14 that were selected (6 PV [2 homozygous, 4 heterozygous for V617F JAK2], 4 IE & 4 normals) were chosen on the basis that enough erythroblasts (>0.4 million) with >95% purity had been produced to yield sufficient (>5ug) high quality RNA (A260/280 = 1.9-2.1) following Qiagen extraction as described in 2.7.1. Biotinylated fragmented RNA was derived from this primary RNA using the rigorous approach outlined in 2.7.2-3 applied identically to all samples.

Quality control procedures carried out on the fragmented cRNA using the Agilent Bioanalyser showed samples were of good quality. These samples were then hybridised onto U133a GeneChips as discussed in 2.7.4. U133a GeneChips offer a broad expression profile including that of 14,500 well characterised human genes. Hybridisation of the biotinylated erythroblast RNA onto these arrays gave consistent GeneChip exposures as shown in Figure 1[6].

Figure 1[6]: Boxplots showing consistent chip exposure in the 14 microarrays
(PV = Polycythaemia Vera, IE = Idiopathic Erythrocytosis, N = Normals)



6.1.2 Statistical analysis of microarrays

Data analysis of the microarrays was carried out using the R statistical environment and programming language [Venables & Ripley, 2002] and Bioconductor packages [Gentleman et al, 2004]. The 'affy' package written to handle Affymetrix data, and specifically the 'rma' algorithm for pre-processing, normalizing and calculation of expression values was used [Irrizary et al, 2003]. Heatmaps were created using the Cluster and Treeview software packages [Eisen et al, 1998, Mootha et al, 2003].

Classical multidimensional scaling (or principle coordinates method) was used to show the relation between patient samples. This transformed the high dimensional expression signature differences between samples into an optimum 2-dimensional descriptive figure with the minimum of distortion.

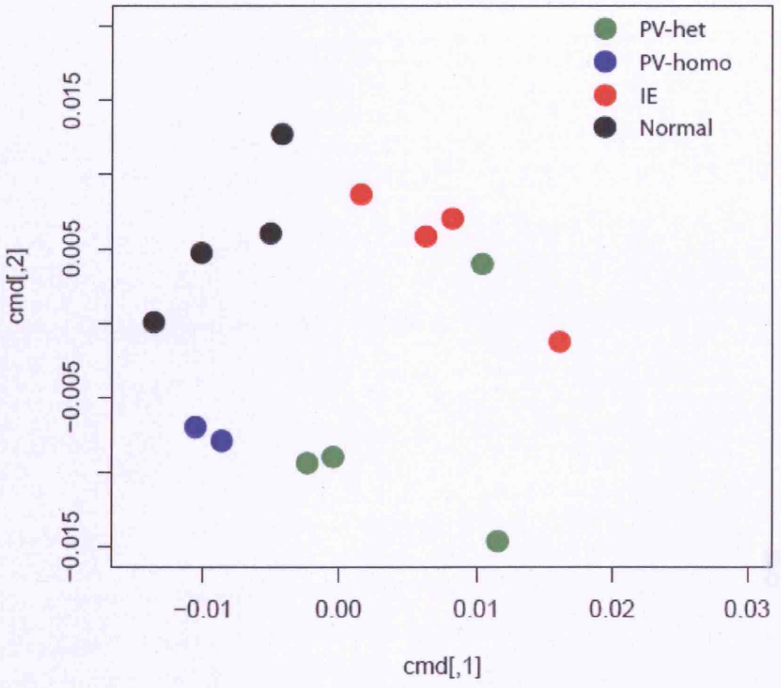
The gene enrichment analysis used a resampling method (n=10000), to calculate the whether the 'Jak/STAT' and 'differentiation' genesets were significantly modulated above the null distribution. Ten thousand randomly extracted genesets were extracted from the data, and the sum of t-values recorded for each. The significance (p) value was then the fraction of t-value sums which exceed the t-value sum of the Jak/STAT or differentiation set.

6.1.3 Segregation of microarray signature by subject group

Analysis of the microarray data showed that there was clear separation between the PV, IE and normal groups. Classical multidimensional scaling (as described in 2.7.5) was used to illustrate the relation between patient samples and this additionally showed separation of the PV subjects homozygous & heterozygous for V617F JAK2 (Figure 2[6]). One of the samples, from a patient with PV who was heterozygous for V617F JAK2, was an outlier for some of the differentiating genes but segregated with the PV patients for other differentiating genes. This patient did not show any significant differences from other PV patients in clinical phenotype, however, there was evidence of mild myelofibrosis on her bone marrow trephine.

Figure 2[6]: Multidimensional scaling analysis of the microarray data shows separation of the subject groups

(analysis of 14 GeneChips including 4 PV patients with homozygous [PV-homo] & 2 with heterozygous [PV-het] V617F JAK2, 4 patients with Idiopathic erythrocytosis [IE] & 4 normal subjects all expressing wild type JAK2)



Comparison was made between samples from patients with PV and the 2 control groups and genes showing the most significant dysregulation are listed in Table 1[6]. Downregulation of genes was more frequently observed in PV than upregulation. A heatmap of the 96 most differentially expressed genes comparing the 3 subject groups is shown in Figure 3[6]. This and subsequent heatmaps are depicted with increasing intensity of red reflecting gene overexpression & increasing intensity of green reflecting gene underexpression relative to the group as a whole.

Table 1[6]: Genes with altered expression on U133a microarrays in PV

Genes showing marked differentiation between PV & control groups	Fold change
Upregulation	
interferon induced transmembrane protein 1 (9-27)	3.59
interferon induced transmembrane protein 1 (9-27)	2.96
insulin receptor substrate 2	1.79
KIAA0876 protein	1.43
pim-1 oncogene	1.69
genomic large histone family cluster	1.49
histone 1, H1c	1.49
hypothetical protein LOC151162	1.37
Downregulation	
BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	0.34
guanine nucleotide binding protein (G protein), alpha 13	0.36
protein tyrosine phosphatase, receptor type, O	0.37
butyrate-induced transcript 1	0.43
ribonucleotide reductase M1 polypeptide	0.46
cyclin A2	0.51
cell division cycle 27	0.52
protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	0.54
cAMP responsive element binding protein 1	0.55
adenosylmethionine decarboxylase 1	0.56
TPX2, microtubule-associated protein homolog (Xenopus laevis)	0.57
microtubule-associated protein 7	0.58
protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	0.59
microtubule-associated protein 7	0.60
cell division cycle 27	0.60
dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	0.60
LanC lantibiotic synthetase component C-like 1 (bacterial)	0.60
acid phosphatase 1, soluble	0.61
COP9 signalosome subunit 8	0.62
ribonucleotide reductase M2 polypeptide	0.69
alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	0.77
chromosome 14 open reading frame 136	0.78

Figure 3[6]: Heatmap showing 96 of the most discriminatory genes

(includes 6 PV, 4 IE and 4 normal control samples [N])

6.1.4 Erythroid differentiation assessed by microarray analysis

Sets of genes known to be associated with erythroid differentiation were identified from the scientific literature and are shown in Table 2[6]. The expression pattern of these genes was analysed using the gene enrichment method described in 2.7.5 to assess whether expression of the erythroid differentiation set was modulated significantly from the null distribution for the sample set.

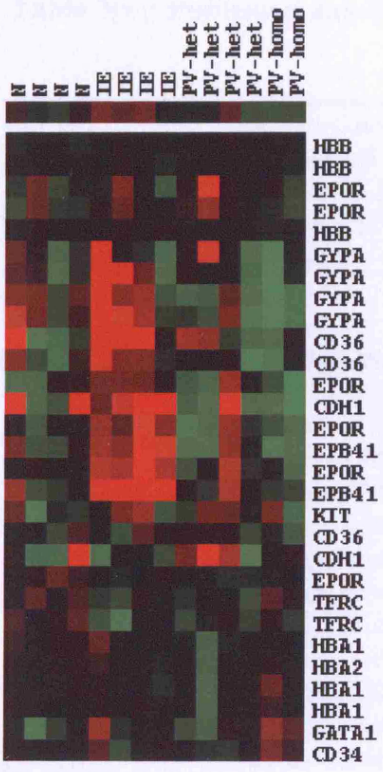
Table 2[6]: Genes known to be associated with erythroid differentiation

CD34	CD34 molecule
CD36	Thrombospondin receptor
KIT	Stem cell receptor (CD117)
GYPA	Glycophorin A
HBB	Hemoglobin beta
HBA1	Hemoglobin alpha
CDH1	E-Cadherin precursor
EPOR	Erythropoietin receptor
GATA1	GATA-binding protein 1
TFRC	Transferrin receptor (CD71)
EBP41	Protein 4.1

Comparison of all groups (PV, IE & normals) showed a significant difference in erythroid differentiation (p=0.0005). However, as can be seen in Figure 4[6], these differences were due to increased expression in the IE group with PV & normal groups showing similar expression patterns. This overall pattern was consistent with the flow cytometric (FACS) assessment of immunophenotype: FACS data showed that IE versus non IE subjects were 56% +/-7% vs. 48% +/-3% Glycophorin A positive (mean fluorescence intensity 26 vs. 24 +/-3) and 84% +/-4% vs. 81% +/-2% CD36 positive (mean fluorescence intensity 29 vs. 18 +/-3).

Figure 4[6]: Heatmap showing gene expression by erythroid differentiation marker in subject groups

(includes data from 4 normal subjects, 4 IE and 6 PV [PV-het & PV-homo denotes PV subjects heterozygous and homozygous for V617F JAK2], red = increased expression, green = decreased expression)



6.1.5 Janus-associated kinase (JAK2) gene expression

With the knowledge of the association between V617F JAK2 and Polycythaemia Vera, JAK2 gene expression was specifically assessed to see whether any differences were apparent. There were 2 probes for JAK2 on the U133a GeneChips. Whilst there was an indication that IE had a marginally higher level of JAK2 expression than normal samples (mean IE:N= 8.39:7.54, p=0.03), an F-test comparison of all 3 groups was not significant (mean IE:PV:N= 8.39:7.64:7.54, p=0.10). The conclusion is therefore that the V617F JAK2 mutation is not associated with an altered level of JAK2 gene expression in erythroblasts.

6.1.6 Candidate targets of Jak-STAT activation

It seemed important to consider carefully whether the differences noted in the microarray data might represent a simple Jak/STAT imprint. In order to explore this, an extensive search was performed to identify genes which had been clearly identified as Jak-STAT targets in published literature and these genes are shown in Table 3[6].

Table 3[6]: Published Jak-STAT targets

BCL2L1	BCL-2 related protein (BCLXL included)
MCL1	Myeloid cell leukaemia 1
BIRC5	Apoptosis inhibitor 4
MYC	Oncogene MYC
CCND1	Cyclin D1
CCND2	Cyclin D2
CCND3	Cyclin D3
TP53	Tumor protein p53
VEGF	Vascular endothelial growth factor
HIFIA	Hypoxia-inducible factor 1, alpha subunit
PIM1	Oncogene PIM1
PIM2	Oncogene PIM2
CISH	Cytokine-inducible SH2-containing protein
SOCS1	Suppressor of cytokine signaling 1
SOCS2	Suppressor of cytokine signaling 2
SOCS3	Suppressor of cytokine signaling 3
SOCS4	Suppressor of cytokine signaling 4
SOCS5	Suppressor of cytokine signaling 5
SOCS6	Suppressor of cytokine signaling 6
SOCS7	Suppressor of cytokine signaling 7
OSM	Oncostatin M
IRF1	Interferon regulatory factor 1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2
IFI27	Interferon-alpha-inducible protein 27
IFI16	Interferon-gamma-inducible protein 16
IFITM1	Interferon-induced transmembrane protein 1
IFITM2	Interferon-induced transmembrane protein 2
IFITM3	Interferon-induced transmembrane protein 3
ARNT	Hypoxia-inducible factor 1, beta subunit
GRB10	Growth factor receptor-bound protein 10
CEBPA	CCAAT/Enhancer-binding protein, alpha
ETS1	Oncogene ETS1

Using the gene enrichment test to compare these genes with other randomly selected genes it was clear that these Jak-STAT targets were significantly modulated against the null distribution overall for PV samples compared to both control groups (p=0.0183). However, within the group of Jak/STAT targets there was a spectrum of modulation with some genes showing no significant differences in expression as illustrated in Figure 5[6].

Figure 5[6]: Heatmap showing gene expression of known Jak-STAT targets (includes evaluation of the published Jak-STAT targets listed in Table 3[6] by subject group, red = increased expression, green = decreased expression)

6.2 PCR validation of basal erythroblast gene expression

Planning validation of the microarray dataset was difficult for two reasons. Firstly, the differences in gene expression detected by the microarray analysis were relatively small fold changes predominantly involving gene downregulation in PV. Secondly, there was a limited quantity of residual high purity primary erythroblast RNA. As it would be difficult to be confident of results with low polymerase chain reaction (PCR) copy number due to reduced expression, genes which were up-regulated in PV were selected for validation. Wherever possible (and unless stated otherwise) the same primary material as had been used for the microarray analysis was used for validation.

A SYBR-green conjugated Taqman approach as outlined in 2.4.7 was used for validation. Selected genes were Pim-1 oncogene (PIM1), Interferon-induced transmembrane protein 1 (IFITM1) Core binding factor, alpha subunit 2, translocated to,3 (CBFA2T3) and Leptin receptor (LEPR). These 4 genes were chosen to include 2 which were believed to be Jak-STAT targets (PIM1, IFITM1), and 2 which were not (LEPR & CBFA2T3). After some initial experiments, Oncogene ABL (ABL) was selected as a single 'housekeeping gene'.

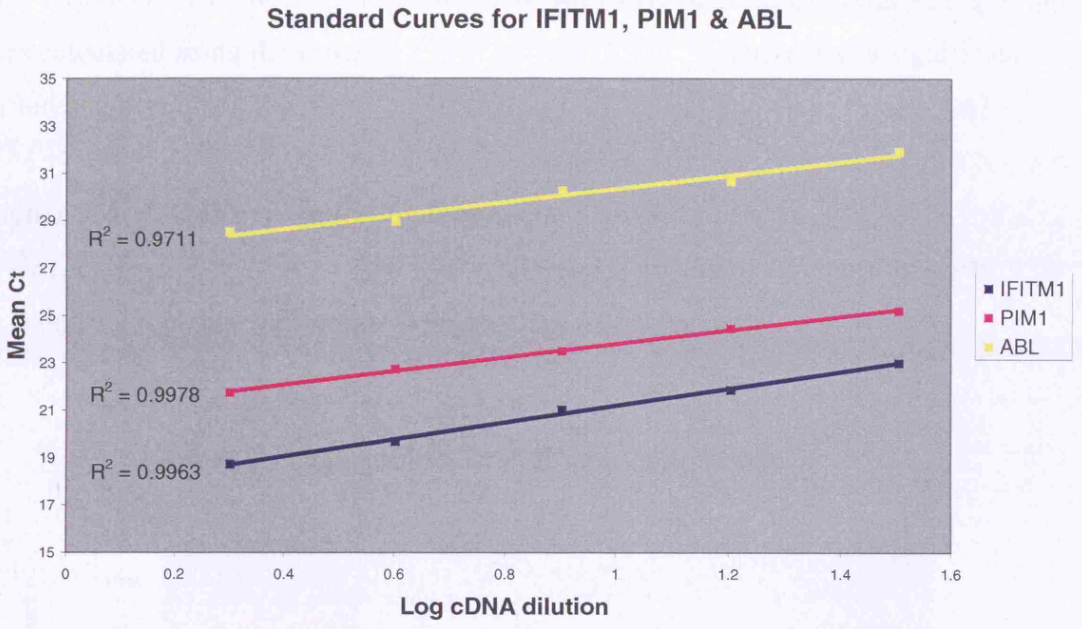
6.2.1 Quality control of real-time quantitative PCR (RQ-PCR) analysis

Taqman primers for PIM1 and ABL were taken from published data [Kim et al, 2005; Beillard et al, 2003] and primers for IFITM1, LEPR and CBFA2T3 were designed 'in house' as there were no suitable published alternatives. RNA from leukaemic cells was used to derive cDNA as per 2.4.3. This cDNA was diluted 1:2, 1:4, 1:8, 1:16 and RQ-PCR performed in triplicate on at least 2 occasions as per 2.4.7. Standard curves were constructed, trendlines applied and R^2 values calculated as shown in Figure 6[6] confirming a parallel relationship between the selected and housekeeping gene within the mean threshold cycle (Ct) range appropriate to subsequent experiments.

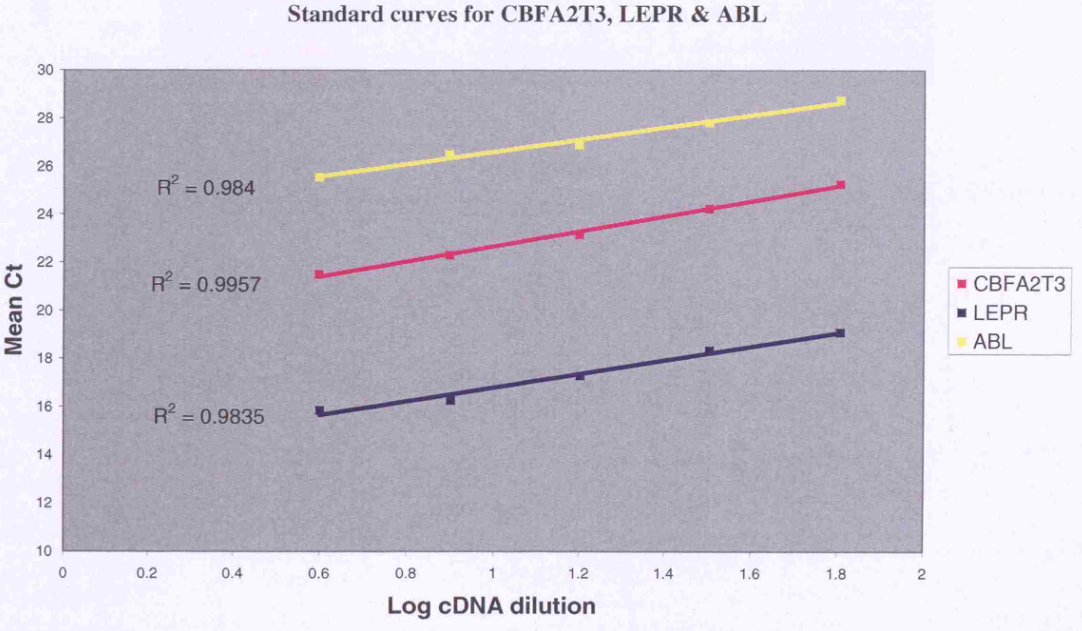
Figure 6[6]: Standard curves for Taqman analysis

(RNA from leukaemic cells reverse transcribed to cDNA, cDNA diluted 1:2, 1:4, 1:8, 1:16 and RQ-PCR performed in triplicate for a) IFITM1, PIM1 & ABL b) CBFA2T3, LEPR & ABL on at least 2 occasions using a SYBR-green Taqman approach, mean threshold cycle [Ct] plotted against log cDNA dilution)

a)



b)

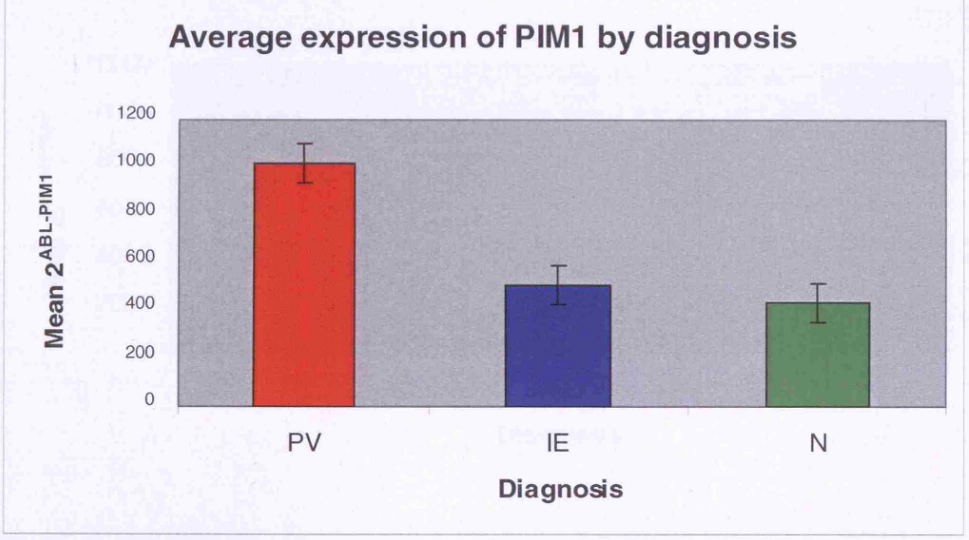


6.2.2 Pim-1 oncogene (PIM1)

Fresh cDNA was prepared from the erythroblast stock RNA on each occasion with cDNA amplified in duplicate for real-time quantitative PCR using SYBR-Green as described in 6.2.1. Sixteen samples were available including 13/14 used in the microarray analysis and 3 further samples of comparable quality (1 IE, 2 Normals). Six/16 samples were processed on 3 separate occasions, 5/16 on 2 separate occasions and 5/16 only once. Mean Ct was calculated for PIM1 & ABL and relative expression was calculated using the formula $2^{(\text{mean Ct ABL - mean Ct PIM1})}$. There was a significant difference between PV patients and the control groups as shown in Figure 7[6].

Figure 7[6]: PIM1 expression by diagnostic group

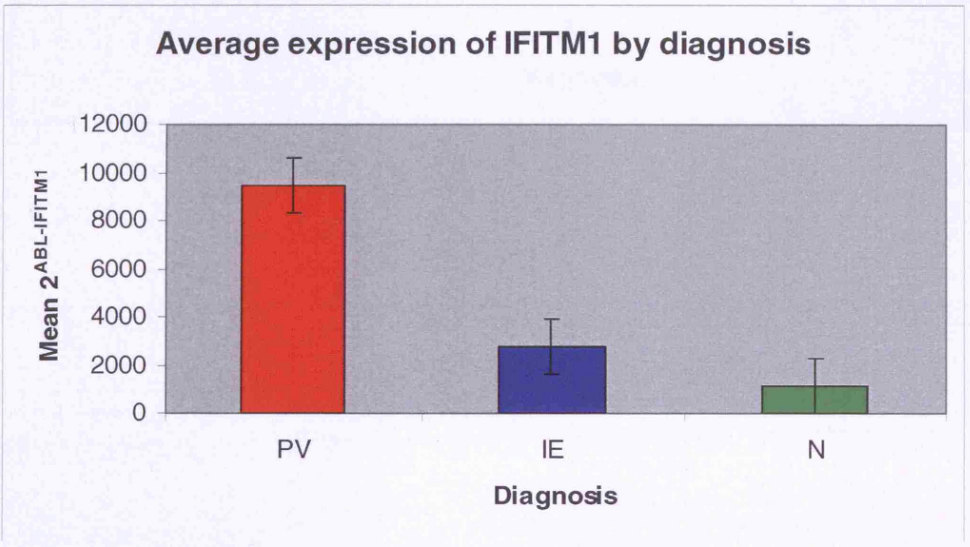
(PIM1 expression relative to ABL shown, includes averaged results from 6 PV, 5 IE & 5 normal subjects with standard error of the mean shown)



6.2.3 Interferon-induced transmembrane protein 1 (IFITM1)

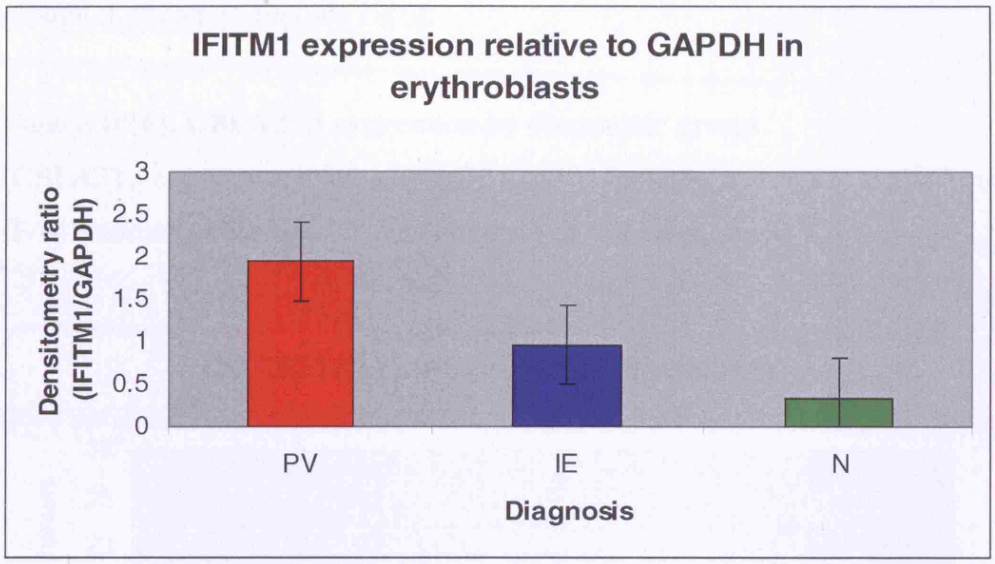
Fresh cDNA was prepared from the erythroblast stock RNA on each occasion with cDNA amplified in duplicate for real-time quantitative PCR using SYBR-Green as described in 6.2.1. Sixteen samples were available including 13/14 used in the microarray analysis and 3 further samples of comparable quality (1 IE, 2 Normals). Eleven/16 samples were processed on 2 separate occasions and 5/16 on 1 occasion. Relative expression was calculated as in the previous section. There was a significant difference between PV patients and the control groups as shown in Figure 8[6].

Figure 8[6]: IFITM1 expression by diagnostic group
(IFITM1 expression relative to ABL shown, includes averaged results from 6 PV, 5 IE & 5 normal subjects with standard error of the mean shown)



As IFITM1 was so strongly over-expressed in the PV subjects a simple semi-quantitative approach was developed to corroborate these findings using different primers. The same erythroblast template RNA was used as in the preceding experiments and cDNA was produced in an identical fashion. cDNA was amplified simultaneously for IFITM1 and GAPDH as described in 2.2.4. Findings confirmed overexpression of IFITM1 in PV as shown in Figure 9[6].

Figure 9[6]: Semi-quantitative analysis of IFITM1 / GAPDH expression (simultaneous amplification of IFITM1 & GAPDH was performed using the same samples of template RNA as in Figure 8[6], resolved products were quantified by densitometry & the ratio of IFITM1 to GAPDH is shown with standard error of the mean plotted)

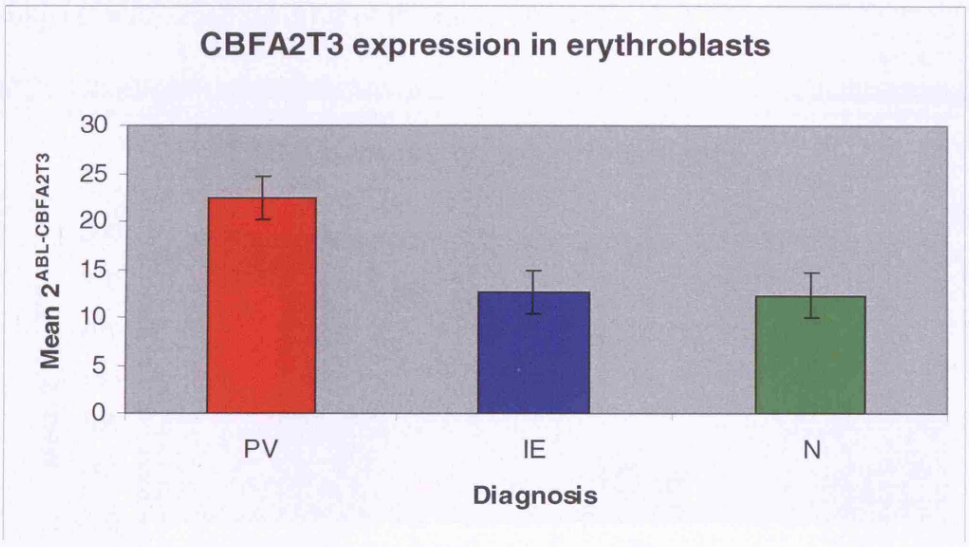


6.2.4 Core binding factor, runt domain, α subunit 2, trans. to 3 (CBFA2T3)

CBFA2T3 expression was evaluated by RQ-PCR in duplicate using freshly made cDNA from 15 subjects. Template erythroblast RNA from these 13 subjects included 13/14 microarray samples and 2 further samples of similar quality from normal subjects. There was a significant difference between PV patients and the control groups as shown in Figures 10[6].

Figure 10[6]: CBFA2T3 expression by diagnostic group

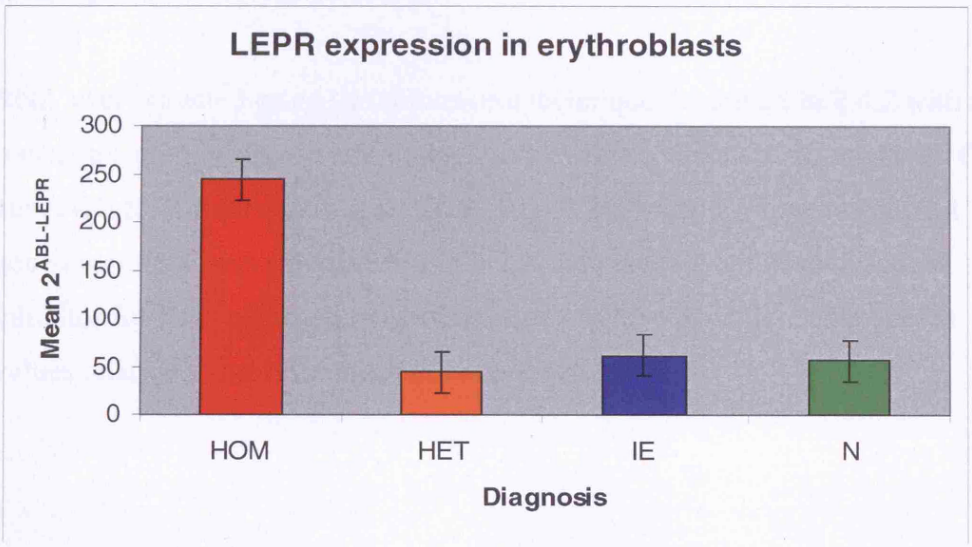
(CBFA2T3 expression relative to ABL shown, includes averaged results from 6 PV, 4 IE & 5 normal subjects with standard error of the mean shown)



6.2.5 Leptin receptor (LEPR)

LEPR expression was evaluated by RQ-PCR using the same samples and methods described in 6.2.4. Using this technique, homozygosity for V617F JAK2 was the factor which resulted in the significant increase in LEPR expression found in PV as shown in Figure 11[6]. This finding was not apparent in the microarray analysis where expression was portrayed relative to the whole sample set (samples homozygous for V617F JAK2 in lanes 3 & 5 of the heatmap shown in Figure 3[6]).

Figure 11[6]: LEPR expression by diagnostic group & V617F JAK2 status
(LEPR expression relative to ABL shown, includes averaged results from 6 PV [2 HOM = homozygous, 4 HET = heterozygous for V617F JAK2, 4 IE & 5 normal subjects with standard error of the mean shown)



6.3 PCR analysis of the modulatory effects on gene expression of inhibitors of JAK2 in haematopoietic cell lines and primary erythroblasts

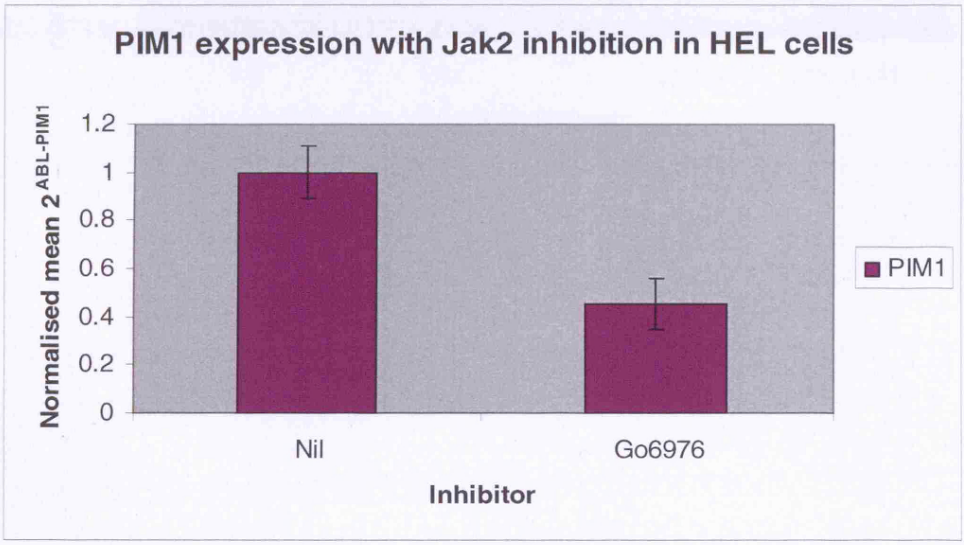
Having confirmed the upregulation of PIM1, IFITM1, CBFA2T3 & LEPR in erythroblasts, further work was done to explore whether this increased expression was the result of overactive JAK signalling. Experiments on haematopoietic cell lines were performed using HEL cells (cytokine independent, homozygous expression of V617F JAK2) and TF1 cells (cytokine dependent, expressing wild type Jak 2). Experiments on primary cells were performed using CD34+ derived erythroblasts from normal individuals and Day 9 erythroblasts derived from the peripheral blood of patients with PV & IE. Cytokine dependent cells were washed and starved, then pre-incubated with either Go6976 or Jak Inhibitor 1 followed by stimulation with erythropoietin as described in 2.6.1.

RNA was extracted using the chloroform technique described in 2.4.2 with reverse transcription as in 2.4.3. Semi-quantitative evaluation was performed with GAPDH as per 2.4.4. Real-time quantitative PCR (RQ-PCR) was performed as per 2.4.7. Relative gene expression was calculated as in 6.2.2 and results were normalised (to no inhibitor for HEL cells and to cytokine alone for cytokine dependent cells). Mean values relative to the normaliser are expressed below.

6.3.1 PIM1

Five separate experiments were performed where HEL cells were exposed to the JAK2 inhibitor, Go6796. The effects of this inhibitor were evaluated semi-quantitatively initially but differences were subtle and difficult to quantitate, so an RQ-PCR approach was adopted. Results are shown in Figure 12[6] and suggest that PIM1 expression in HEL cells may be JAK2 dependent. This effect was also apparent at a protein level as shown in Figure 19[4].

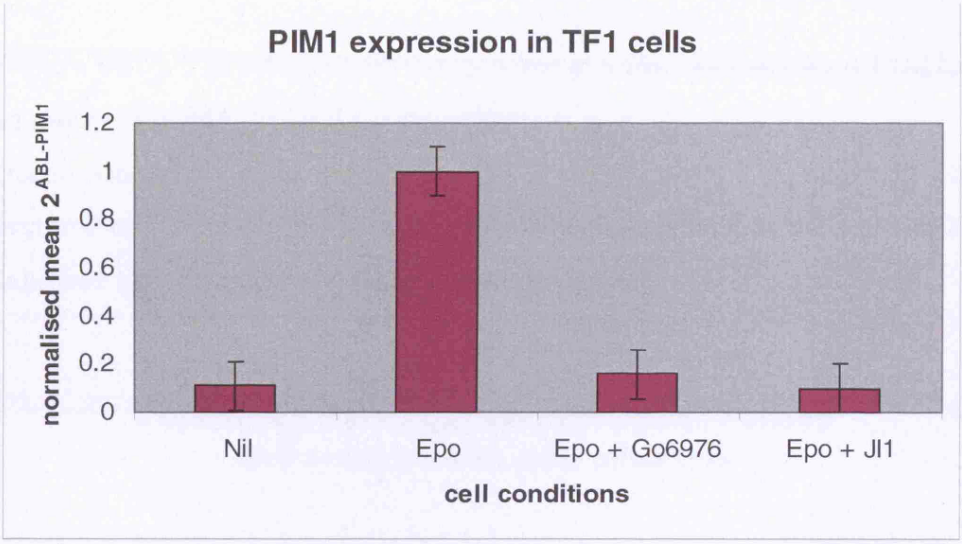
Figure 12[6]: The effect of JAK2 inhibition on PIM1 expression in HEL cells (mean normalised results from 5 experiments where HEL cells were incubated with no inhibitor or Go6976 for 6 hours, standard error of the mean shown)



In 4 experiments TF1 cells were starved and then stimulated with erythropoietin for 1 hour +/- pre-incubation with Go6976 or Jak Inhibitor 1. PIM1 expression was evaluated by RQ-PCR and the results are presented in Figure 13[6] showing that PIM1 expression is erythropoietin inducible in a JAK2 dependent manner in these cells. This pattern was also noted with Go6976 pre-incubation & GM-CSF stimulation.

Figure 13[6]: The effect of erythropoietin & JAK2 inhibition on PIM1 expression in TF1 cells

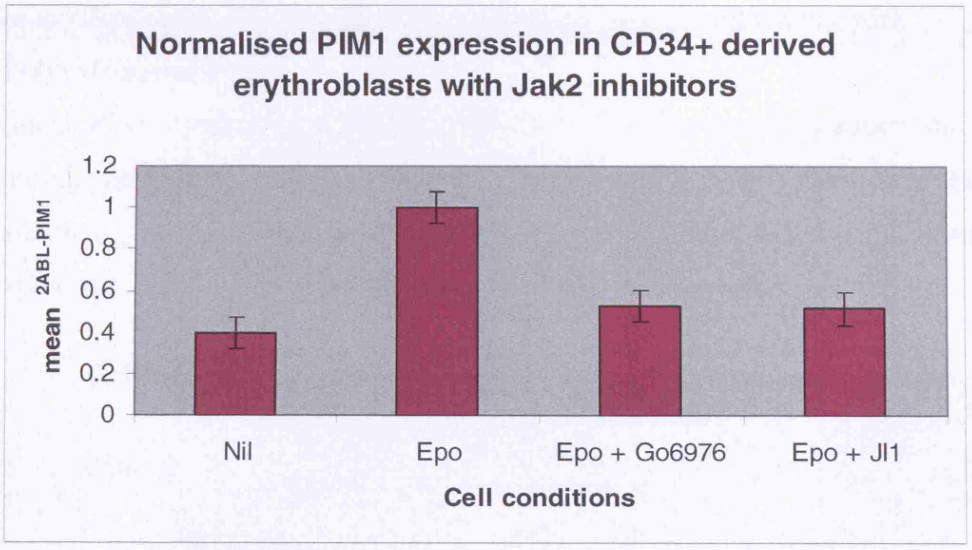
(mean normalised PIM1 expression relative to ABL from 4 experiments where TF1 cells were starved and then stimulated with erythropoietin -/+ Go6976 or Jak Inhibitor 1[JI1], standard error of the mean shown)



Five experiments were performed using CD34+ derived erythroblasts which had been washed and starved for 2 hours and then stimulated for 1 hour with erythropoietin +/- pre-incubation with Go6976 or Jak Inhibitor 1. Results from these experiments are shown in Figure 14[6] and confirm that PIM1 expression is erythropoietin induced and JAK2 dependent in normal erythroblasts. This pattern persisted at a 4 hour stimulation time point.

Figure 14[6]: The effect of erythropoietin & JAK2 inhibition on PIM1 expression in normal CD34+ derived erythroblasts

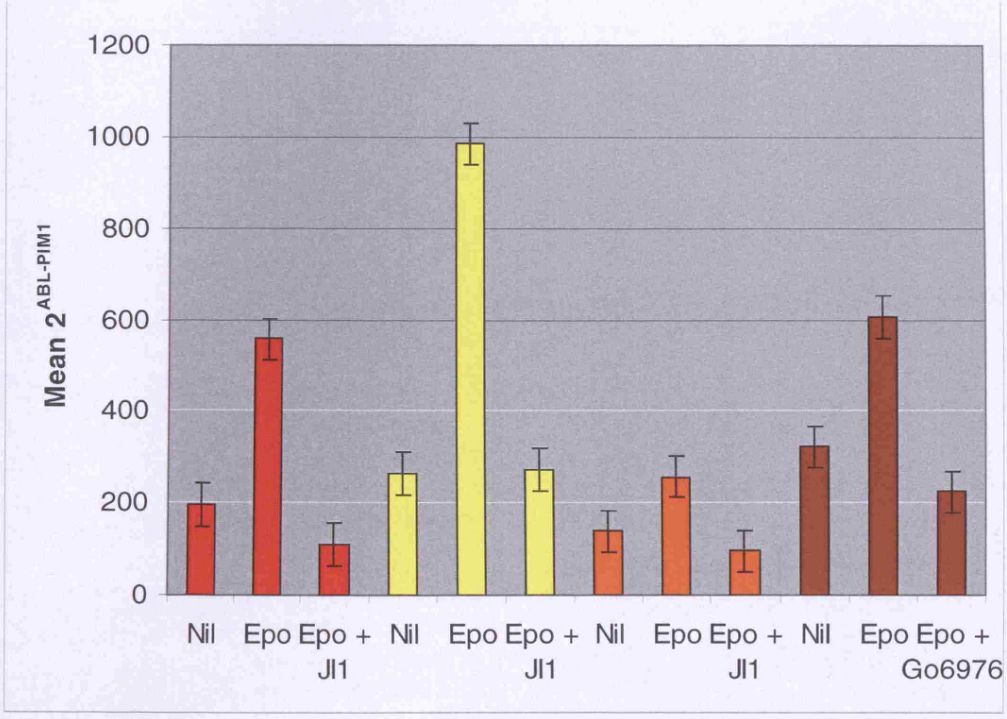
(mean normalised PIM1 expression relative to ABL from 5 experiments where erythroblasts were starved & then stimulated with erythropoietin -/+ Go6976 or Jak Inhibitor 1[JI1], standard error of the mean shown)



Four experiments were performed using erythroblasts derived from the peripheral blood of patients with PV (1homozyous, 3 heterozygous for V617F JAK2). RNA was extracted from cells which had been washed and starved for 2 hours and then stimulated with erythropoietin +/- pre-incubation with Jak Inhibitor 1 (n=3) or Go6976 (n=1). As previously, fresh derived cDNA was amplified in duplicate. Three/4 primary samples were analysed on 3 occasions, 1/4 on 2 occasions. Mean results from these experiments are shown in Figure 19[6] and confirm that PIM1 expression is erythropoietin induced and JAK2 dependent in PV erythroblasts. It can also be seen that there is a trend to reduction of PIM1 levels to below basal expression with JAK2 inhibition as was seen in protein analysis in primary erythroid cells shown in Figure 22[4].

Figure 15[6]: The effect of erythropoietin & JAK2 inhibition on PIM1 expression in erythroblasts derived from the peripheral blood of patients with Polycythaemia Vera

(mean PIM1 expression relative to ABL from experiments in 4 patients shown (1 homozygous [red], 3 heterozygous for V617F JAK2), erythroblasts were starved [Nil] and then stimulated with erythropoietin [Epo] -/+ pre-incubation with Jak Inhibitor 1[JI1, n=3] or Go6976, standard error of the mean shown)

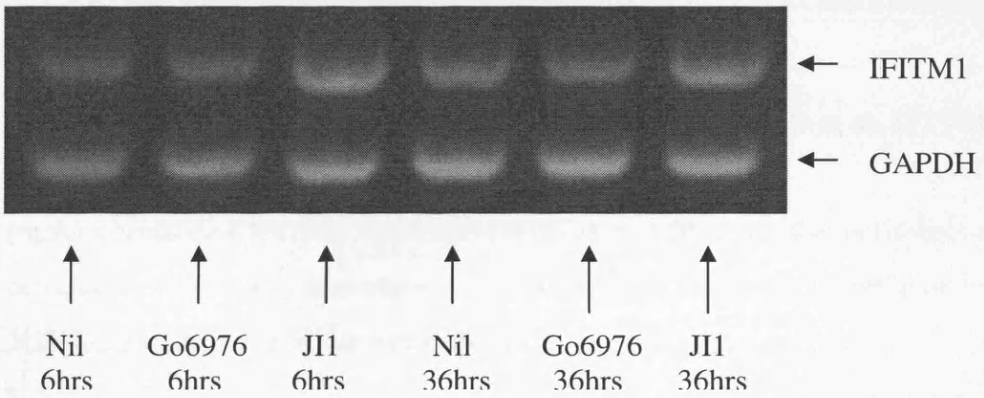


6.3.2 IFITM1

Initial experiments were performed using HEL cell derived RNA following exposure to the Jak 2 inhibitors Go6976 and Jak Inhibitor 1 followed by semi-quantitative evaluation of IFITM1 with GAPDH. Good products were obtained but repeat experiments showed no significant effect of JAK2 inhibition visually or by densitometry and an example of this is shown in Figure 16[6].

Figure 16[6]: Semi-quantitative analysis of IFITM1 with GAPDH using RNA extracted from HEL cells exposed to JAK2 inhibitors

(RNA was extracted from HEL cells exposed to either Go6976 or Jak Inhibitor 1 [JI1], cDNA was amplified for IFITM1 & GAPDH simultaneously and products resolved on an agarose gel)



As IFITM1 is an interferon response gene known to be induced by Jak/STAT signalling, this lack of response to JAK2 inhibitors seemed surprising and so the effect of these inhibitors was further assessed by RQ-PCR. These experiments were performed simultaneously with the same primary samples and subsequent analysis described in the previous section for PIM1 (results shown in Figures 12[6] – 15[6]). The derived results are shown below in Figures 17[6] = 20[6] and confirm that IFITM1 expression is not JAK2 dependent in HEL cells and is not erythropoietin-induced in a JAK2 dependent manner in TF1 cells, normal or PV erythroblasts.

Figure 17[6]: The effect of JAK2 inhibitors on IFITM1 expression in HEL cells

(mean normalised IFITM1 expression relative to ABL from 5 experiments where HEL cells were incubated with no inhibitor or either Go6976 or Jak Inhibitor 1 [JI1] for 6 hours, standard error of the mean shown)

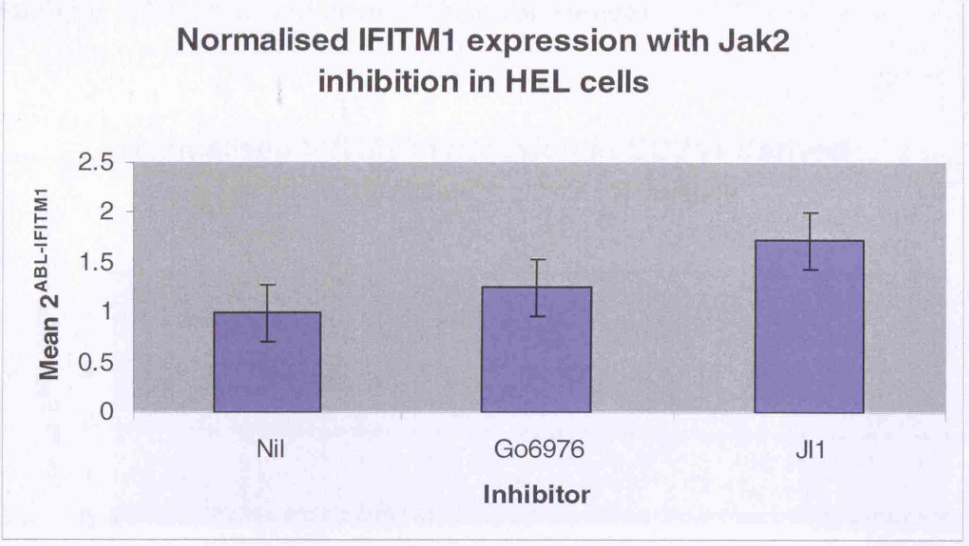


Figure 18[6]: The effect of erythropoietin & JAK2 inhibition on IFITM1 expression in TF1 cells

(mean normalised IFITM1 expression relative to ABL from 4 experiments where TF1 cells were starved and then stimulated with erythropoietin -/+ Go6976 or Jak Inhibitor 1[JI1], standard error of the mean shown)

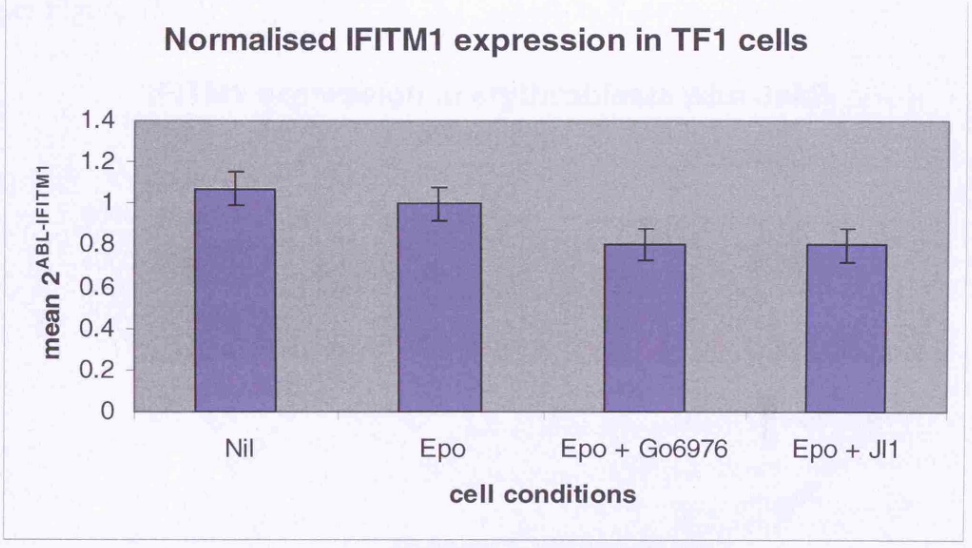


Figure 19[6]: The effect of erythropoietin & JAK2 inhibition on IFITM1 expression in normal CD34+ derived erythroblasts

(mean normalised IFITM1 expression relative to ABL from 5 experiments where erythroblasts were starved and then stimulated with erythropoietin -/+ Go6976 or Jak Inhibitor 1[JI1], standard error of the mean shown)

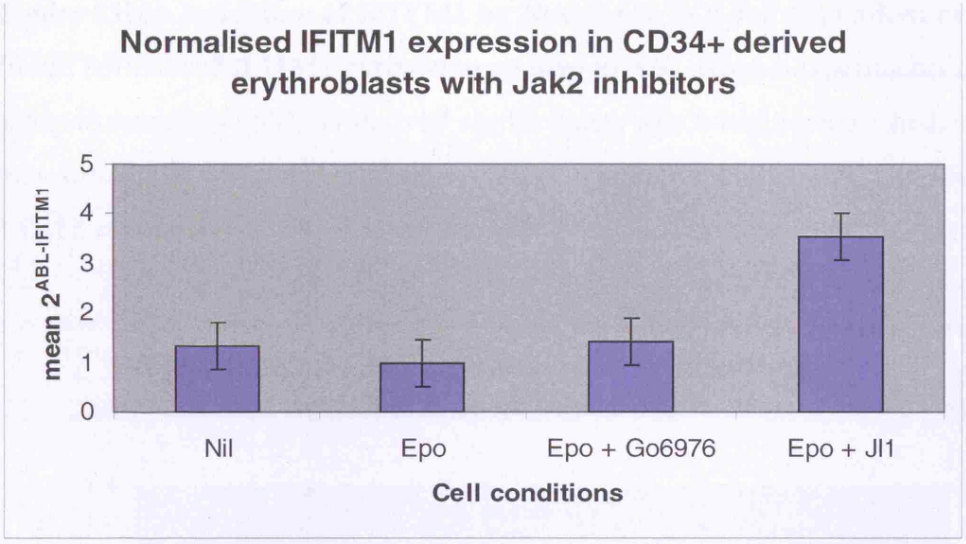
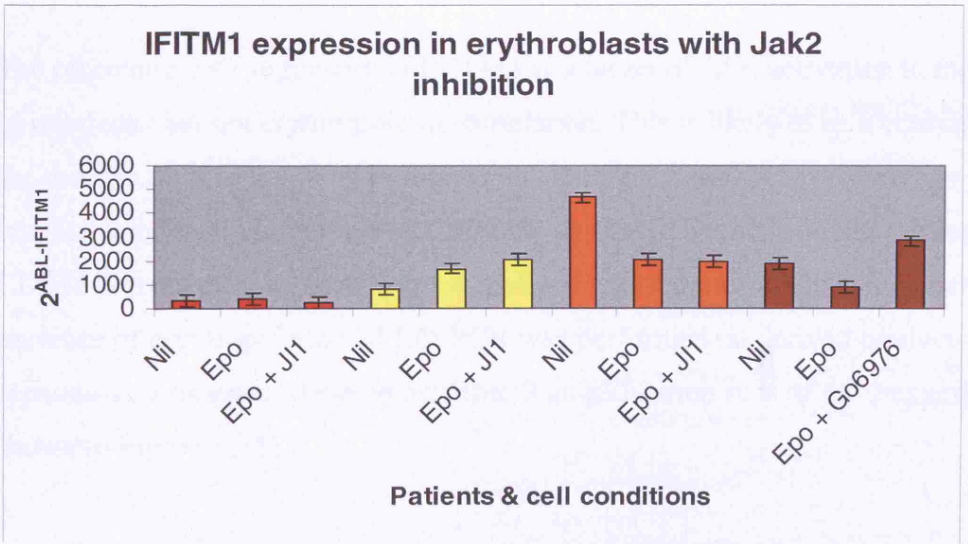


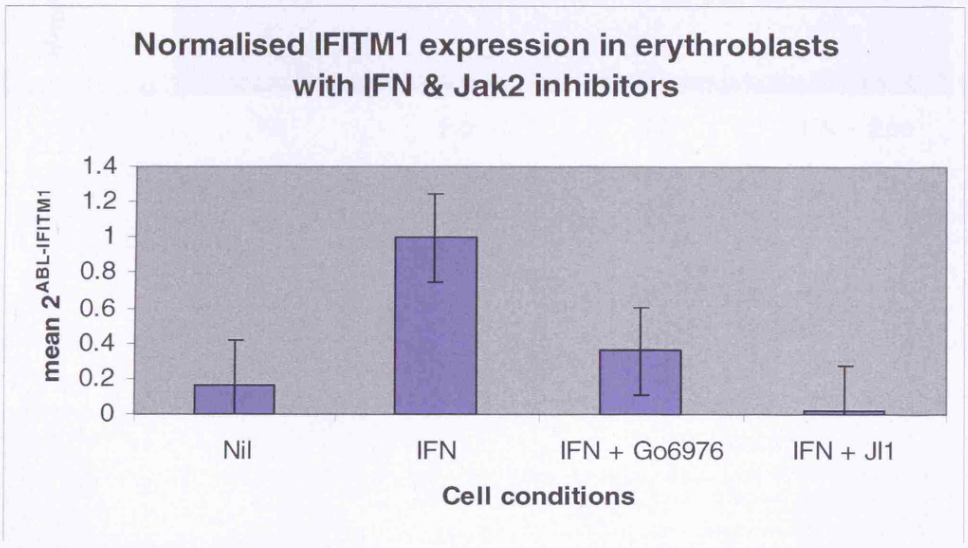
Figure 20[6]: The effect of erythropoietin & JAK2 inhibition on IFITM1 expression in erythroblasts derived from the peripheral blood of patients with Polycythaemia Vera

(mean IFITM1 expression relative to ABL from experiments in 4 patients shown as per Figure 15[6])



Whilst investigating IFITM1 expression in TF1 cells it was shown that IFITM1 expression was interferon-inducible in these cells. Further experiments were performed to assess the effects of interferon stimulation +/- JAK2 inhibition using CD34+ derived erythroblasts and it was found that interferon stimulation of IFITM1 was Jak dependent in these cells as shown in Figure 21[6].

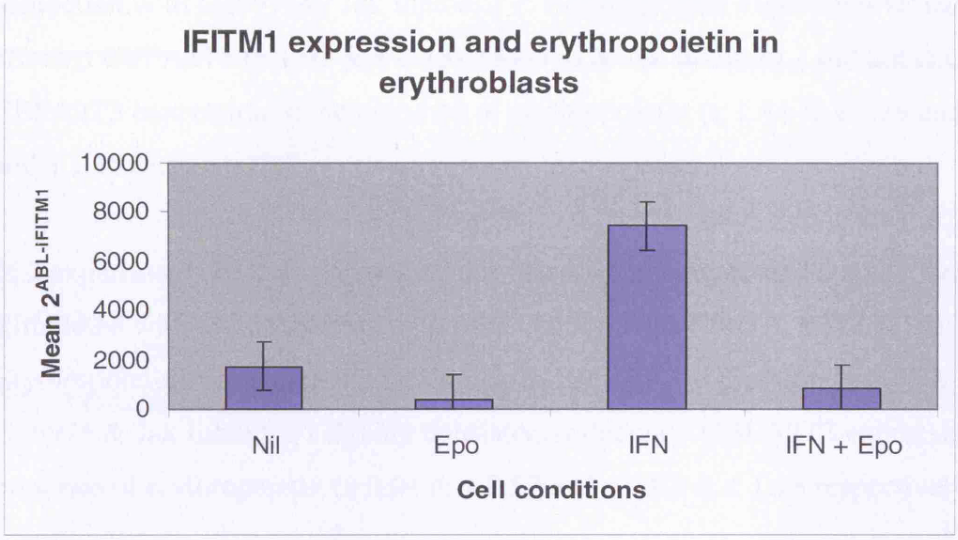
Figure 21[6]: Induction of IFITM1 by Interferon in a Jak dependent manner (mean normalised IFITM1 expression relative to ABL from 4 experiments using 2 separate samples of CD34+ derived erythroblasts which had been washed, starved and then stimulated with Interferon alpha -/+ pre-incubation with Go6976 or Jak Inhibitor 1 [JI1], standard error of the mean shown)



The preceding data suggests that IFITM1 is a target of JAK activation in the context of interferon but not erythropoietin stimulation. This is likely to be a consequence of the specific STATs involved in interferon signalling. Furthermore, Epo can inhibit the interferon induced upregulation of IFITM1 as shown by the following experiments. CD34+ derived erythroblasts were stimulated with Interferon alpha in the absence or presence of erythropoietin and RQ-PCR was performed on derived products from 2 separate experiments. Mean values from 3 amplification runs of the 2 experiments are shown in Figure 22[6].

Figure 22[6]: The inhibitory effect of erythropoietin on IFITM1 expression in CD34+ derived erythroblasts

(mean values from 3 amplification runs of 2 experiments using CD34+ derived erythroblasts -/+ Interferon alpha -/+ Erythropoietin, standard error of the mean shown)



6.3.3 CBFA2T3

Two experiments were performed where HEL cells were exposed to Go6976 and Jak Inhibitor 1 for 4 hours. Go6976 reduced expression of CBFA2T3 (x 0.73 & x 0.40 whilst Jak Inhibitor 1 had variable effects (x 2.17 & x 0.56). In 2 experiments TF1 cells were starved and then stimulated with erythropoietin with or without pre-incubation with Go6976 or Jak Inhibitor 1. Erythropoietin expression variably affected CBFA2T3 (x 1.01 & x 6.43). Go6976 & Jak Inhibitor 1 did not decrease CBFA2T3 expression in the presence of erythropoietin (x 1.44 & x 1.29 and x 3.67 and x 1.15 respectively).

In 2 experiments CD34+ derived erythroblasts were washed and starved then stimulated with erythropoietin +/- pre-incubation with Go6976 and Jak Inhibitor 1. Erythropoietin stimulation did not increase CBFA2T3 expression (x 0.05 & x 0.86). Go6976 & Jak Inhibitor 1 did not consistently decrease CBFA2T3 expression in the presence of erythropoietin (x 0.04 & x 0.57 and x 0.02 & x 1.18 respectively).

The experimental material from PV erythroblasts used in 6.1.3 & 6.2.3 and portrayed in Figures 15[6] and 20[26] was also tested in parallel with LEPR for CBFA2T3 expression. Fresh cDNA was amplified in duplicate on 2 separate occasions. There was no clear relationship with erythropoietin stimulation or JAK2 inhibition as shown in Figure 23[6] for 3/4 patients. Mean values from normalised results are shown in Figure 24[6] and confirm this overall impression. These results are inconclusive, but suggest that CBFA2T3 is not dependent on the erythropoietin – Jak/STAT axis.

Figure 23[6]: Results from 4 separate experiments using erythroblasts from patients with PV exploring the effect of JAK2 inhibition on CBFA2T3 expression

(mean CBFA2T3 expression relative to ABL in 4 experiments using peripheral blood derived erythroblasts from patients with PV which were stimulated with Epo -/+ pre-incubation with Go6976 or Jak Inhibitor 1 [JI1])

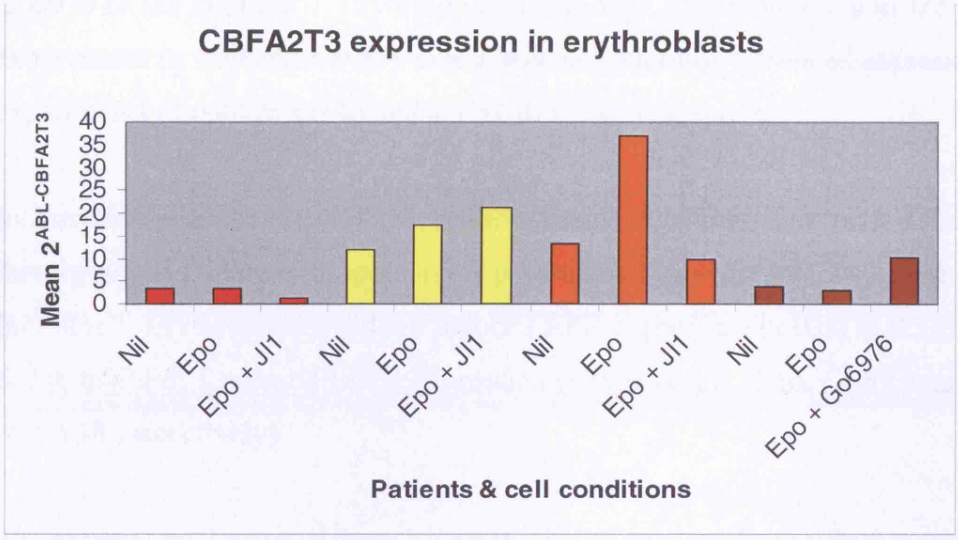
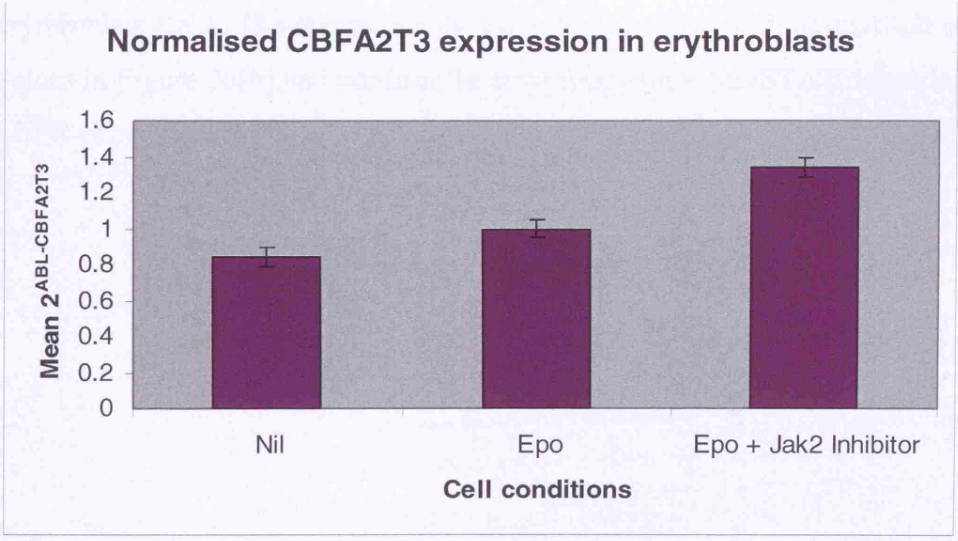


Figure 24[6]: Mean normalised CBFA2T3 expression in erythroblasts with erythropoietin stimulation and JAK2 inhibition

(normalised mean results from Figure 23[6], standard error of the mean shown)



6.3.4 LEPR

Two experiments were performed where HEL cells were exposed to Go6976 and Jak Inhibitor 1. Neither Go6976 nor Jak Inhibitor 1 consistently reduced LEPR expression (x 2.5 & x 2.9 and x 1.42 & x 0.57 respectively). In 2 experiments TF1 cells were starved and then stimulated with erythropoietin with or without pre-incubation with Go6976 or Jak Inhibitor 1. Erythropoietin induced LEPR expression in 1/2 experiments (x 1.29 and x 0.27). Go6976 & Jak Inhibitor 1 reduced expression in 3/4 experiments (x 0.40 & x 0.44 and x 1.82 & x 0.43 respectively).

In 2 experiments CD34+ derived erythroblasts were washed and starved for 2 hours then stimulated with erythropoietin +/- pre-incubation with Go6976 2.5uM or Jak Inhibitor 1. Erythropoietin did not induce LEPR expression (x 0.02 & 0.33). Go6976 & Jak Inhibitor 1 reduced LEPR expression (x 0.02 & x 0.35 for Go6976 and x 0.01 & x 0.18 respectively).

The experimental material from PV erythroblasts used in the previous section was also tested in parallel with CBFA2T3 for LEPR expression with fresh cDNA amplified in duplicate on 2 separate occasions. LEPR expression was increased with erythropoietin stimulation and was reduced with JAK2 inhibition in each case. The single patient homozygous for V617F JAK2 had markedly higher levels of LEPR expression as had been noted previously by RQ-PCR of the microarray template erythroblast RNA. The results are shown in Figure 25[6] with normalised mean values in Figure 26[6] and confirm the erythropoietin – Jak/STAT dependency of LEPR expression in PV erythroblasts.

Figure 25[6]: Results from 4 separate experiments using erythroblasts from patients with PV exploring the effect of JAK2 inhibition on LEPR expression

(LEPR expression relative to ABL in 4 experiments using peripheral blood derived erythroblasts from patients with PV which were stimulated with Epo -/+ pre-incubation with Go6976 or Jak Inhibitor 1 [JI1])

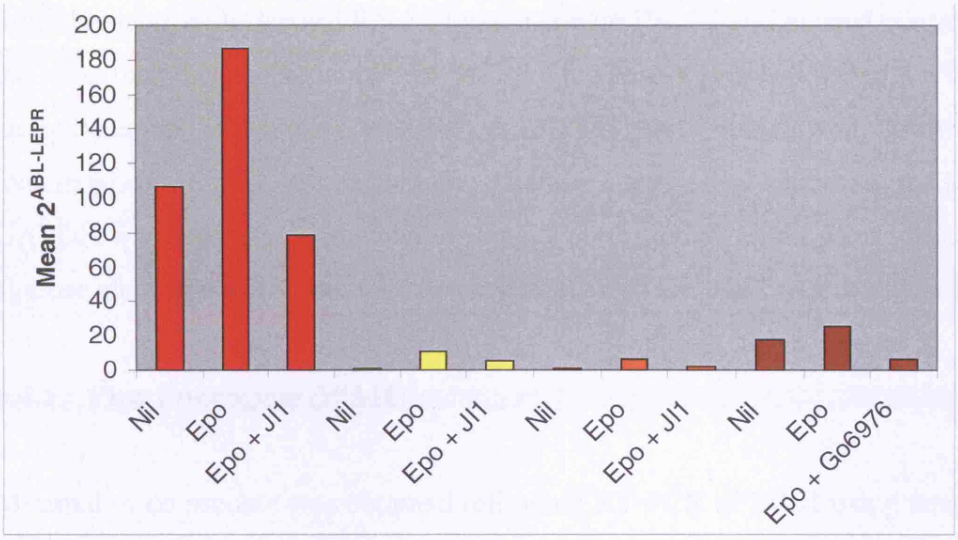
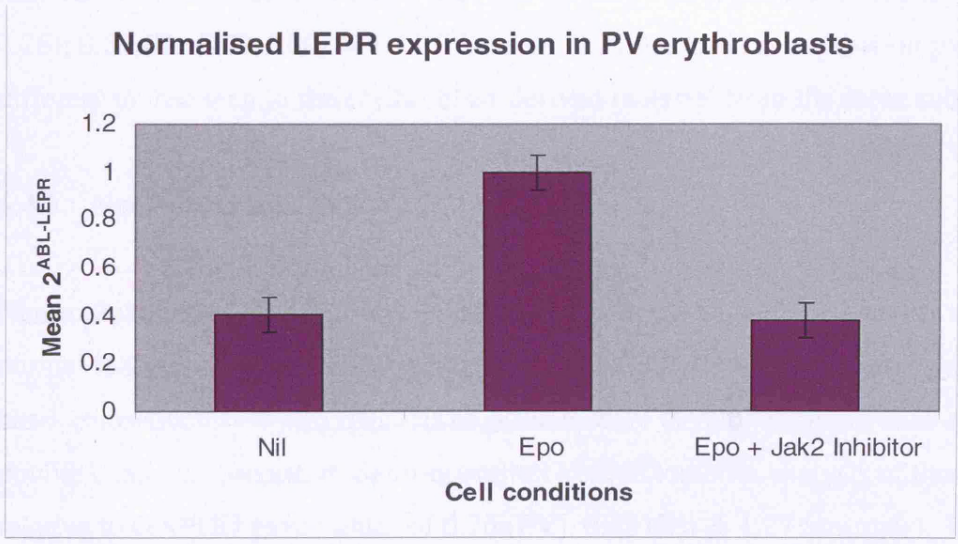


Figure 26[6]: Mean normalised LEPR expression in erythroblasts with erythropoietin stimulation and JAK2 inhibition

(normalised mean results from Figure 25[6], standard error of the mean shown)



6.4 Semi-quantitative analysis of gene expression using RNA derived from neutrophils

Since neutrophils are easier to collect than erythroblasts it might have been useful to identify changes in neutrophil gene expression for further investigative work. Four genes were selected and assessed using semi-quantitative amplication of cDNA made from the neutrophil-derived RNA of patients with PV, IE and normal controls using the same methods as that employed for PRV1. These 4 genes included 2 which were upregulated on the microarrays (PIM1 & IFITM1) and 2 which were downregulated (Nucleophosmin [NP1] & Protein phosphatase 2 regulatory subunit alpha [PPP2R2A]. GAPDH was used as a 'housekeeping' gene, amplified products were resolved with agarose electrophoresis and band density relative to GAPDH was calculated.

6.4.1 Pim-1 oncogene (PIM1)

Minimal or no product was obtained following RT-PCR of PIM1 using neutrophil derived RNA.

6.4.2 Interferon induced transmembrane protein 1 (IFITM1)

Expression was assessed in 8 patients with PV, 5 patients with IE and 7 normal individuals. Mean densitometric ratios for IFITM1 / GAPDH were 1.08 (PV, 0.63-2.28), 0.69 (IE, 0.39-1.00) & 1.11 (Normals, 0.74-1.38). This expression pattern was different to that seen in the erythroblast-derived material from the same subjects.

6.4.3 Nucleophosmin (NP1)

Neutrophil derived material was assessed in 6 patients with PV, 4 patients with IE & 4 normal individuals. The PCR product with the initial primer design gave a double band. Subsequently 4 different sets of primers were developed but in each case a double band was produced. Semi-quantitative densitometric analysis of these bands relative to GAPDH gave values of 0.76 (PV), 0.45 (IE) & 1.27 (normals). This pattern of relative expression differs from that noted in the erythroblast microarrays.

6.4.4 Protein phosphatase 2 regulatory subunit alpha (PPP2R2A)

Neutrophil derived material was assessed in 6 patients with PV, 4 patients with IE & 4 normal individuals. Semi-quantitative densitometric analysis relative to GAPDH gave values of 0.19 for PV, & 0.53 for IE. The faint products for PPP2R2A formed from normal samples were not suitable for densitometric analysis. These neutrophil-derived results are not, therefore, comparable with the expression pattern in erythroblasts in that normals had lower expression than PV.

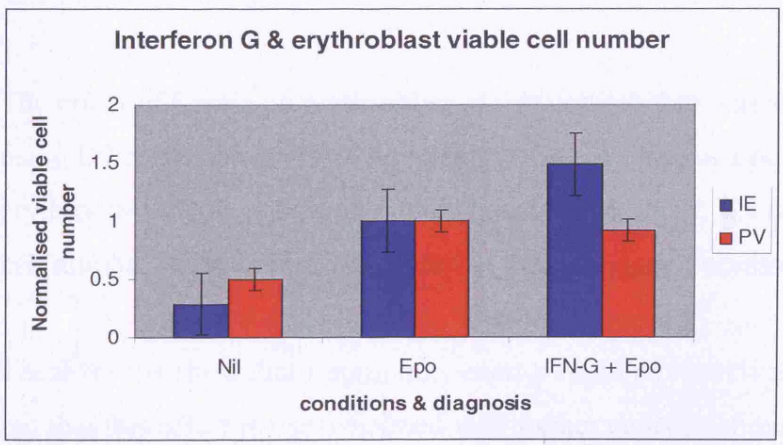
6.5 The contribution of Interferons & Leptin to erythroblast proliferation & survival

6.5.1 Interferons

IFITM1 is a known interferon response gene. In the knowledge that this was upregulated in PV, further work was done to assess the affects of Interferon in primary cells. Interferon Alpha (IFN-A) or Gamma (IFN-G) was introduced into colony assays to assess their effects on erythroid proliferation. Averaged results from 7 (IFN-A) & 8 (IFN-G) experiments gave non-significant reductions in BFUe formation relative to controls of 12% & 22%. EECs were reduced with both agents in a single experiment.

Interferon Gamma was introduced into 5 MTS assays using Day 9 erythroblasts (3 PV, 2 IE) and produced a slight increase in erythroblast viable cell number with some differences between PV & IE as shown in Figure 27[6]. One experiment was performed where the effects of IFN-A, B & G were evaluated in parallel by MTS assay and with Annexin V (AV = Annexin V negative cells) staining & FACS analysis. The ratio of viable cells to the erythropoietin only controls were 1.1 (AV = 0.9) for IFN-A, 1.5 (AV = 1.3) for IFN-B & 1.9 (AV=1.6) for IFN-G. These results confirm the complex effects of interferons on erythroblast viable cell number with anti-proliferative and anti-apoptotic impact.

Figure 27[6]: Average results from 5 experiments assessing the effects of Interferon-G (IFN-G) on erythroblast viable cell number

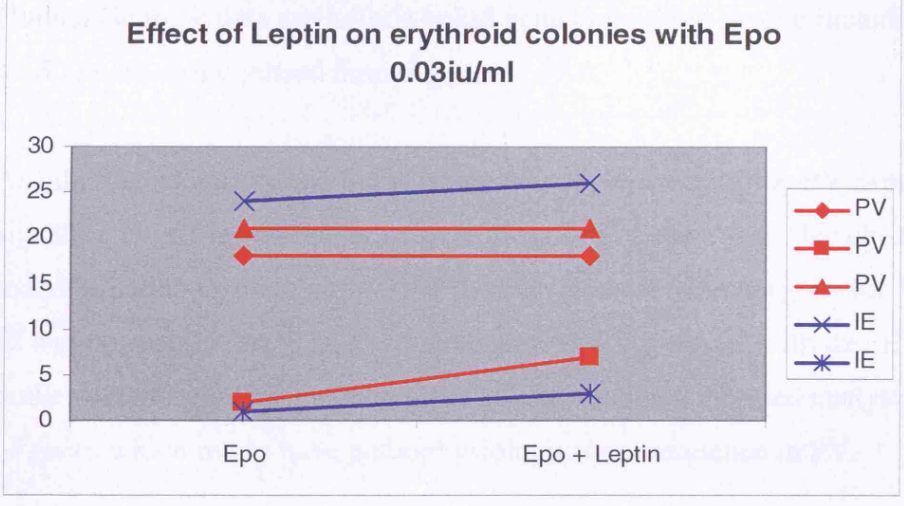


6.5.2 Leptin

Once upregulation of LEPR was ascertained in PV it was felt that some preliminary work assessing the effects of Leptin on primary cells would be relevant. Recombinant human Leptin was introduced into colony assays to assess its effect on erythroid proliferation. Averaged results from 7 experiments showed a non-significant increase of 14% in colony formation relative to controls. In order to explore the effect of Leptin at more physiological concentrations of erythropoietin, Epo was restricted to 0.03iu/ml in 5 colony assays with or without Leptin. The results shown in Figure 28[6] suggest that Leptin makes little contribution to erythroblast proliferation.

Figure 28[6]: Results from 5 experiments where Leptin was added to erythroid colonies with low level erythropoietin

(colony assays from 3 patients with PV & 2 with IE with Epo 0.03iu/ml +/- Leptin 200ng/ml)



The effect of Leptin on erythroblast viable cell number was assessed by MTS assay using D9 erythroblasts from 3 patients. In the absence of Epo Leptin increased erythroblast viable cell number by a mean factor of 1.2. There was no effect on viable cell number when Leptin was added in the presence of erythropoietin 2iu/ml.

These results show that Leptin may exert a slight pro-survival effect on erythroblasts but that this effect is overwhelmed with higher concentrations of added erythropoietin.

6.6 Discussion

Whilst there is some literature on the gene expression profile of neutrophils and CD34+ cells from patients with PV, there is no published data on erythroid progenitor gene expression in this disease. The work presented in this chapter was designed to compare erythroid gene expression between patients with clonal (PV) and non-clonal (IE) erythroid expansion as well as normal controls. This gave insight into the changes in gene expression which simply result from erythroid expansion and those which provide clues to an underlying myeloproliferative disorder.

Template RNA was extracted from highly purified erythroblasts derived from the peripheral blood of 14 individuals including 6 with PV (2 homozygous, 4 heterozygous for V617F JAK2) 4 with IE & 4 normal subjects (all wild type for JAK2). Fragmented biotinylated RNA derived from this template material was hybridised onto U133A microarrays with evidence of good quality control. Affymetrix U133A GeneChips were produced following publication of the draft Human Genome data and offer a broad gene expression profile including that of 14,500 well characterised human genes.

As other investigators had found, there was segregation of the PV expression signature from the control signatures. Intriguingly, there was also clustering of each individual diagnostic category (PV [homozygous & heterozygous for V617F JAK2], IE and normals). Only 1 of the 6 patients with PV grouped with the IE samples. As an outlier overall, this PV patient helped to narrow down targeted analysis of the group of genes which might have pathophysiological consequence in PV.

The altered expression profile found in PV was broad but subtle in keeping with the slow pace of this disease relative to acute leukaemia in vivo. Comparing PV to the control subjects, the most upregulated gene was IFITM1 with a 3.59 fold increase. Gene downregulation was more frequently observed in PV but was less marked, with the significant fold decreases reaching a minimum of 0.34 for BUB1.

It is recognized that the gene expression pattern of erythroblasts varies with the number of days of in vitro culture as progenitors differentiate. [Scicchitano et al, 2003] For this reason, all the samples in this study were analysed after the same period of in vitro erythroblast culture. A possible explanation for the altered expression profile observed in PV observed in this study was primary differences in PV erythroblast differentiation. The published data on this issue is inconclusive. One group of investigators observed earlier and increased expression of Glycophorin A in CD34+ derived liquid cultures from the bone marrow of patients with PV. [Ugo et al, 2004] By contrast, other groups which used CD34+ cells derived from peripheral blood did not find altered differentiation in PV erythroblasts [Dai et al, 2005; Zeuner et al, 2006].

Work presented in Chapter 3 did not show altered erythroid differentiation in peripheral blood – derived PV erythroblasts by standard immunophenotyping and FACS analysis. Erythroid differentiation was again assessed using the microarray dataset and confirmed these findings. It was however noteworthy, (as had been the case with the immunophenotyping data) that the IE samples showed small but statistically significant differentiation differences from the PV and normal groups. There is no immediate explanation for these findings.

A key issue in analysis of these microarrays was whether the altered gene expression profiles seen in PV represented a V617F JAK2 imprint. Levels of JAK2 mRNA expression were initially assessed as one group of investigators had found increased levels of JAK2 mRNA derived from neutrophils in PV and IE compared to normal controls. [Lippert et al, 2006] There was no increased JAK2 expression in PV erythroblasts in this study.

An extensive literature search was performed to identify genes which had previously been implicated as Jak/STAT targets. Identified Jak/STAT targets broadly incuded suppressors of cytokine signalling (SOCS), interferon-inducible genes, known oncogenes such as ETS1, TP53, BCL-2 & Pim-1, hypoxia inducible genes such as ARNT, HIF1A & VEGF, anti-apoptotic targets such as MCL1, BIRC5 & BCL2L1 & D-Cyclins [Clarkson et al, 2006; Hartman et al, 2005 ; Nelson et al, 2004; Bromberg

et al, 2000 ;Der et al, 1998 ; Zeng et al, 2006 ; Klampfer et al, 2003 ; Geiss et al, 2003 ; Xu et al, 2005 ; Stout et al, 2005 & Numata et al, 2005].

The dataset was then interrogated for significant alteration in expression of these genes compared to other randomly selected genes using the gene enrichment method. The Jak/STAT target genes overall were significantly modulated in PV (p=0.0183), however, there was a spectrum of modulation with some genes known to be Jak/STAT targets showing no altered expression in PV. It is possible that the supraphysiological levels of erythropoietin used in the culture system employed in this study may have 'smoothed out' the effects of an aberrant V617F Jak/STAT imprint. However, this 'smoothing' effect may also have given a selection advantage for differential expression of genes which are independent of Jak/STAT and this dataset may therefore give new insight into the pathophysiology of PV beyond V617F JAK2 expression.

The microarray data was validated by performing real-time quantitative PCR amplification of 4 genes which were upregulated in the PV microarrays using predominantly the same template erythroblast RNA as had been used in the microarray experiments. Four genes were selected to include 2 genes believed to be Jak/STAT targets (PIM1 & IFITM1) and 2 genes which were not (CBFA2T3 & LEPR) so that further work could be done to assess the JAK2 dependency of these genes by use of JAK2 inhibitors. As had been the case in the microarray analysis, whilst there was individual sample variation, overexpression of these 4 genes in PV erythroblasts was confirmed. Markedly increased expression of LEPR in the PV patients homozygous for V617F JAK2 was also observed. Having identified dysregulation of key genes in PV erythroid progenitors the JAK2 dependency of these 4 genes was then explored with use of specific JAK2 inhibitors.

Oncogene PIM1 is located at 6p21.1 and encodes for a serine/threonine protein kinase. Dysregulation or mutation of PIM1 has been described in several human cancers including leukaemias, lymphomas and solid tumours. PIM1 is well recognized as a STAT target. [Matikainen et al, 1999] Overexpression of PIM1 has also been shown to independently contribute to tumour development by induction of genomic

instability [Roh et al, 2003] and to induce cytokine independence in haematopoietic cell lines. [Nosaka et al, 2002]

In 1993 it was reported that PIM1 deficient mice exhibited erythrocyte microcytosis and that overexpression of PIM1 in mice induced erythrocyte macrocytosis on the background of an otherwise normal phenotype. [Laird et al, 1993] The precise mechanism for the role of Pim-1 in erythropoiesis is unknown, however the recent finding of a functional association between Pim-1 and ribosomal protein S19 (implicated in the ineffective erythroid progenitor maturation observed in Diamond-Blackfan anaemia) may pave the way for an improved understanding of this process. [Orru et al, 2006].

The microarray data and RQ-PCR findings presented in this chapter showed aberrant overexpression of PIM1 in PV erythroid progenitors. Using specific inhibitors of JAK2, analysis showed that PIM1 was Jak/STAT dependent in tumour cells expressing wild type or V617F JAK2. It was then found that PIM1 was erythropoietin inducible in a JAK2 dependent fashion in primary erythroblasts from normal individuals expressing wild type JAK2 and from patients with PV expressing V617F JAK2. It therefore seems likely that upregulated PIM1 in PV erythroblasts represents a V617F JAK2 imprint. The subsequent lack of a PIM1 PCR product from the PV neutrophil RNA was unsurprising since PIM1 is expressed at low levels in circulating adult neutrophils.

Interferon induced transmembrane protein 1 (IFITM1) is located on chromosome 11 & is an interferon-inducible gene whose expression is known to correlate with the sensitivity of cell lines to growth suppression by interferon. Interferon response genes were identified as Jak/STAT targets in 1994. [Darnell et al, 1994] The role of IFITM1 in haemopoiesis was unclear but latterly its' relevance to the migration and differentiation of primordial germ cells has been elucidated. [Tanaka et al, 2005] IFITM1 upregulation has been noted in the CD34+ cells of Myelodysplastic Syndrome (MDS) [Pellagatti et al, 2006] and also in the buffy coat of low risk patients with CML [Aykerli et al, 2005]. Increased IFITM1 expression in MDS & CML cannot, however, be regarded as an aberrant V617F imprint since this mutation

is an infrequent finding in these diseases. [Steensma et al, 2005; Kronenwett et al, 2006]

IFITM1 was significantly upregulated in PV erythroblasts by microarray analysis and RQ-PCR. Use of specific inhibitors of JAK2 showed that IFITM1 was not directly JAK2 dependent in HEL cells expressing V617F JAK2 or in TF1 cells induced by erythropoietin. This effect was also noted in primary and PV erythroblasts which had been stimulated with erythropoietin.

Further work confirmed that IFITM1 was interferon-inducible in a Jak dependent fashion in tumour cells and normal erythroblasts as expected. It was subsequently observed that both starved and interferon-stimulated expression of IFITM1 in erythroblasts was reduced in the presence of erythropoietin. The conclusion is therefore that whilst IFITM1 is an interferon-inducible gene in erythroid progenitors, increased expression of IFITM1 in PV is not the direct result of a V617F JAK2 imprint.

IFITM1 associates with various molecules including CD19, CD21 & CD81 in primary B-lymphocytes and ligation with an antibody imparts an inhibitory signal [Bradbuy et al, 1992]. Therefore, it is perhaps surprising that IFITM1 should be overexpressed in a variety of tumours, including haematological malignancies and colorectal carcinoma. However, the data in germ cells show that IFITM1 has interferon-independent functions and its precise role may vary according to cell context.

Core binding factor, alpha subunit 2, translocated to, 3 (CBFA2T3) is located at 16q24. CBFA2T3 was identified through its association with the t(16,21) translocation found in some patients with acute myeloid leukaemia. [Gamou et al, 1998] Loss of heterozygosity of 16q is a frequent finding in breast cancer and variation of expression of CBFA2T3 in these cells has led to the suggestion that CBFA2T3 is a tumour suppressor gene. [Powell et al, 2002] There is no evidence to suggest that CBFA2T3 is Jak/STAT dependent.

Overexpression of CBFA2T3 in primary PV erythroblasts was shown in this chapter by microarray analysis and confirmed by RQ-PCR. After some investigative work in tumour cells and primary erythroblasts, further study showed that CBFA2T3 was not Jak/STAT dependent in PV erythroblasts and that therefore its increased expression in PV was unlikely to represent a V617F JAK2 imprint.

Leptin was originally described as a hormone that regulated adipose-tissue mass through hypothalamic effects on satiety and energy expenditure. Leptin acts through the leptin receptor (LEPR, encoded at 1p31), a member of the cytokine receptor family. LEPR was initially found to be expressed on haematopoietic progenitor cells [Gainsford et al, 1996]. LEPR expression was later detected on blast cells from patients with AML, ALL and CML and it was shown that leptin could enhance blast proliferation. [Nakao et al, 1998; Konoplova et al, 1999] Most recently, increased LEPR expression has been described in association with chronic phase CML. [Diaz-Blanco et al, 2007]. Whilst LEPR had not been described as a Jak/STAT target it is known that leptin signals via Jak/STAT activation. [Darnell et al, 1994]

Increased expression of LEPR in PV erythroblasts with marked increase in the 2 individuals with homozygous expression of V617F JAK2 was observed in this study. Experiments using JAK2 inhibitors in cell lines were inconclusive but suggested JAK2 dependency of LEPR. Work in normal and PV erythroblasts confirmed that LEPR expression was erythropoietin inducible in a JAK2 dependent manner in these cells. It therefore seems likely that the increased LEPR expression in PV erythroblasts is an effect of aberrant V617F JAK2 signalling with some suggestion of a dosage effect for mutant JAK2. As had been shown by others, work in this study showed a slight enhancement of erythroid proliferation with recombinant human leptin and it is possible that overexpression of LEPR may be one of the mechanisms by which V617F JAK2 increases erythroid proliferation.

A novel gene which showed consistent dysregulation in PV might offer an additional diagnostic tool. It would clearly not be practical to derive erythroblasts from patients for diagnostic purposes so this study also looked at whether the altered expression noted in erythoblasts might be reflected in neutrophils. Neutrophils are plentiful in

peripheral blood and so there was ample neutrophil RNA which allowed exploration of 2 genes which were downregulated in PV; Nucleophosmin (NP1) and Protein phosphatase 2 regulatory subunit alpha (PPP2R2A) as well as PIM1 and IFITM1. Nucleophosmin and PP2R2A were selected as NP1 has already been implicated in the pathophysiology of myeloid disease [Naoe et al, 2006; Grisendi et al, 2006] and phosphatase activity is known to be altered in myeloproliferative disease [Xu et al, 2003]. Preliminary data suggested that findings in neutrophil derived RNA did not correlate with findings in erythroblast derived RNA, so this approach was not pursued.

In summary, this chapter provides unique data on the basic gene expression profile of erythroblasts in Polycythaemia Vera compared with IE and normal subjects. Further analysis of gene expression in the presence of specific inhibitors of JAK2 suggests that this profile is not simply due to a V617F JAK2 imprint since, whilst PIM1 & LEPR over-expression were identified as JAK2 dependent, IFITM1 & CBFA2T3 were found to be JAK2 independent.

CHAPTER 7 – CONCLUSIONS

Polycythaemia Vera is a primary proliferative disorder with leukaemic potential resulting from clonal change in haematopoietic stem cells. In 2005 seminal work was published establishing a link between PV and a specific point mutation in the JAK2 gene. This mutation is not the only clonal change seen in PV although it is the most common. The pathophysiological mechanisms underlying these clonal changes are not well understood. This thesis contributes to understanding of PV by exploring the functional and molecular changes observed in primary erythroid progenitors which result in the increased red cell population which defines PV. It goes on to explore the potential role for small molecule inhibitors as disease modifying agents.

A cohort of patients with true polycythaemia including 27 with PV were characterised clinically and pathologically by EEC formation, V617F JAK2 expression and other parameters. Mononuclear cells were derived from the peripheral blood of patients with PV, Idiopathic Erythrocytosis and normal controls. In standard semi-solid cultures there was no increase in erythroid colonies between PV and IE, although both were increased relative to normals. When these mononuclear cells were established in liquid culture which preferentially selected for erythroid differentiation and growth, qualitatively similar progenitors were derived as defined by morphological and immunophenotypic criteria. However, the quantity of progenitors derived in PV liquid cultures was higher than that seen in control subjects.

When erythroid progenitors were deprived of erythropoietin overnight, PV cells showed relatively sustained viability compared to controls. This feature showed an allele dose response effect in that PV progenitors homozygous for V617F were more sustainable with erythropoietin deprivation than their heterozygous counterparts. This effect was subtle and only became apparent after a large cohort of patients had been studied.

Study of intracellular signal transduction in erythroid progenitors by western blotting showed that there was subtle upregulation of the PI3K, MAPK & Jak/STAT pathways in PV. This effect was apparent with both cytokine deprivation and cytokine

stimulation and, in the case of the PI3K pathway, was sustained over long experimental periods. Increased signal for the Jak/STAT target, PIM-1, was also apparent in PV. Introduction of small molecule inhibitors of PI3K or JAK2 effectively downregulated this increased signalling activity.

When the JAK2 inhibitors, Go6976 & Jak Inhibitor 1, were introduced into colony assays it became apparent that erythropoietin independent colonies could be abolished by JAK2 inhibition. The effects of JAK2 inhibitors were not specific to V617F JAK2 mutant cells as erythroid colony formation was reduced in PV and control cells. In fact there was a trend to reduced susceptibility to inhibitors in the PV subjects homozygous for V617F JAK2. Additionally, using Go6976 & Jak Inhibitor 1 there was reduction in myeloid colony formation in PV and controls. In order to accurately quantify these effects, erythroblast viability in the presence of inhibitor was assessed. Viable cell number was found to be increased in PV samples. This, once again, suggests that mutant progenitors may be less susceptible to JAK2 inhibition than their wild type counterparts and that this reduced susceptibility may be more significant for mutant progenitors exhibiting homozygous expression of V617F.

The PI3K inhibitor, LY294002 (also a PIM-1 inhibitor), reduced erythroblast survival in erythroid progenitors with greater effect in PV than controls but this difference was not apparent with the PI3K inhibitor, PI103. Erythroid progenitor viability with PI103 was affected in a dose dependent manner similar to that seen with JAK2 inhibition for both PV and controls. PI103 was also found to reduce erythroid colony formation and EECs in PV. When specific α, β and δ isoform inhibitors of PI3K were introduced into similar systems it became apparent that pan-PI3K inhibition was necessary to produce effects on signalling and survival in erythroid progenitors. Inhibition studies using Rapamycin identified the mTOR pathway as an alternative therapeutic target.

Whilst there is some literature on the gene expression profile of neutrophils and CD34+ cells from patients with PV there has been no published data on erythroid progenitor gene expression in this disease. The gene expression profile of erythroid progenitors derived from patients with PV, IE and normal controls is compared and contrasted in this thesis. This approach gives insight into the changes in gene

expression which simply result from erythroid expansion and those which provide clues to an underlying myeloproliferative disorder.

Erythroid progenitor gene expression was analysed using U133a Affymetrix GeneChips and by real-time quantitative PCR. There was segregation of the PV expression signature with clustering between subject groups and separation between the subjects heterozygous and homozygous for V617F JAK2. The differences between the groups could not be accounted for by altered differentiation as PV cells showed similar modulation of differentiation genes to normal controls.

The microarray data was validated by RT-PCR amplification of 4 genes which had been found to be upregulated in PV; PIM1, IFITM1, CBFA2T3 & LEPR. These genes were further analysed in erythroid progenitors in the presence of the JAK2 inhibitors described previously. PIM1 was found to be erythropoietin inducible in a JAK2 dependent manner in both PV and controls. IFITM1, whilst clearly an interferon-inducible gene in erythroid progenitors was not JAK2 dependent on erythropoietin stimulation. CBFA2T3 was not JAK2 dependent but LEPR was. It was therefore apparent that upregulation of PIM1 & LEPR could be explained by a V617F JAK2 imprint but that upregulation of IFITM1 & CBFA2T3 could not.

In summary the work presented in this thesis has delineated subtle differences in the functional and molecular processes in primary erythroid progenitors derived from patients with Polycythaemia Vera. The therapeutic potential of PI3K or JAK2 inhibitors in PV is shown in primary cells as distinct from the models described in other published work. A gene expression profile is identified in PV progenitors which shows effects of the V617F JAK2 mutation but also opens up other potential targets for future investigation.

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PUBLICATIONS & ABSTRACTS RESULTING FROM THIS WORK

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Go6976 is a potent inhibitor of the JAK 2 and FLT 3 tyrosine kinases with significant activity in primary acute myeloid leukaemia (AML) cells. *British Journal of Haematology* 2006 Sep 4
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Primary erythroblasts from patients with polycythaemia vera (PV) have a distinct gene expression signature from both idiopathic erythrocytosis (IE) and normal controls which shows limited changes in Jak-STAT target genes
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Poster Presentation BSH 2006 (award winning)

Reversal of apoptosis resistance and erythropoietin independent growth of primary erythroid progenitors from patients with polycythaemia vera (PV) by inhibitors of the Jak2 tyrosine kinase
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Poster Presentation BSH 2006

Optimisation of a robust methodology for the identification of JAK2 mutations in patients with meyloproliferative disorders
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